

**Functional analysis of zebrafish innate immune responses to
inflammatory signals**

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Abstract

Injury, infection and tissue malfunction are triggers of inflammation which if not regulated may acquire new characteristics that result in pathological outcomes. Since innate immunity plays a key role in the resolution of acute inflammation knowledge of the regulation of this component of the host response is relevant to understanding processes in disease progression and therefore has potential clinical benefits.

In this thesis I have applied zebrafish as a model organism to investigate the response of innate immune cells to qualitatively distinct inflammatory signals in the absence of adaptive immunity. Using a zebrafish embryo wound injury model I have investigated leukocyte migration profiles by *in vivo* imaging. In response to wound alone leukocytes migrated to the site of injury with predominantly random walk behaviour. However, the addition of lipopolysaccharide (LPS) enhanced recruitment and influenced the directionality of leukocyte migration to the wound. I demonstrate that leukocyte dynamic behaviour is also dependent on the location of the cells. The LPS enhanced directionality and reduced the random walk behaviour of the leukocytes, and these effects were ablated in the presence of the p38 mitogen-activated protein kinase (MAPK) specific inhibitor SB203580.

Cytokine gene profiling in adult zebrafish leukocytes reveals that LPS can stimulate a pro-inflammatory response via the activation of p38 MAPK characteristic of mammalian innate immune responses.

It is documented in mammalian innate immune cells that LPS can modulate Notch mediated signalling and thereby cell function. Using zebrafish with null mutations in Notch, which provide an unbiased *in vivo* model, I have investigated the influence of Notch signalling on leukocyte recruitment and demonstrate that migration to a wound injury is reduced. However, this effect is due to decreased cell numbers and not altered function as the Notch signalling inhibitor DAPT had no effect of recruitment to wound injury. The defect in myelomonocyte numbers was also present in adult zebrafish and this was partially compensated for by an increase in lymphocytes.

The experimental results that I report here highlight zebrafish as a model

organism for studying the function and regulation of innate immunity. The unique optical translucency, which permits *in vivo* imaging of host responses in real-time, facilitates the analysis of the innate immune response to different inflammatory signals and immune modulators.

Declaration

I declare that the work in this thesis is my own and original except where indicated by special reference in the text. The thesis has not been presented to any other university for examination either in the United Kingdom or overseas.

SIGNED:(HARRIET TAYLOR)

DATE:.....

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Publications

Bugeon, L., Taylor, HB., Ellis, CD., Welsh, N., Smith, E., Vargesson, N., Roberston, BD., Lamb, JR., Dallman, MJ. (Under review). "Notch defects impair myelomonocyte development in zebrafish." Blood.

Controversy persists regarding the role of Notch in myelopoiesis. Previous studies have employed over-expression or loss of function approaches in selected cell lineages. Here we report an unbiased analysis which used two Notch zebrafish mutants beamter (BEA) and deadly seven (DES), with disrupted function in deltaC and notch1a respectively, to analyse leukocyte populations during development and in adults. We found that both BEA and DES adult fish had a decreased proportion of myelomonocytes in both the hematopoietic organ (kidney marrow) and the periphery (coelomic cavity), which was accompanied by an increased proportion of lymphocytes. The myeloid compartment was also affected in the mutant embryos as reflected by lower numbers of granulocytic cells migrating to a wound site. However notch1a mutation did not affect their survival following infection with *Mycobacterium marinum*. Together these results indicate that defects in Notch signalling alter myelopoiesis from the early stages of development.

Taylor, H., Liepe, J., Bugeon, L., Huvet, M., Shia, A., Brown, S., Lamb, JR., Stumpf, MPH., Dallman, MJ. (Submitted). "In-vivo single cell tracking in zebrafish and statistical modelling reveal heterogeneity in recruitment and chemotactic behaviour in leukocytes." J R Soc Interface.

Migration of leukocytes is an important component of the complex inflammatory response to local injury and infection, but the mechanisms of recruitment remain incompletely understood. In this study we use automated computer tracking of single cells in an optically transparent model organism (zebrafish embryo) which provides an unbiased, in vivo, platform for the real-time analysis of leukocyte migration. Using this approach, which increases statistical power in comparison to previous studies, we demonstrate that LPS enhances the recruitment of leukocytes to a wound site. Importantly, LPS influences the directionality, but not the speed of movement of leukocytes in response to wound injury in a p38 mitogen-activated kinase (MAPK)-dependent fashion. We further demonstrate, we believe for the first time in vivo, that leukocyte dynamic behaviour is dependent on the location of cells with respect to blood vessels and the wound site. In particular, random walk-like behaviour is seen to predominate in the proximity of, as well as far away from the site of wound injury, while at intermediate distances, directed migration towards the wound site becomes predominant. This platform offers the opportunity to evaluate the efficacy of candidate modulators that target functional aspects of innate immunity.

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List of Abbreviations

APC	Antigen presenting cell
COX	Cyclooxygenase
Csflr	Colony stimulating factor 1 receptor
CXCL	Chemokine (C-X-C motif) ligand
DC	Dendritic cell
dpf	Days post fertilisation
dpi	Days post injection
ERK	Extracellular-signal-regulated kinase
hpf	Hours post fertilisation
hpi	Hours post injection
ICAM	Inter-Cellular Adhesion Molecule
IL	Interleukin
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
mpo	Myeloperoxidase
PAMPs	Pathogen-associated molecular patterns
PRRs	Pathogen recognition receptors
RA	Rheumatoid Arthritis
TLR	Toll-like Receptor
TNF	Tumour necrosis factor
VCAM	Vascular Cell Adhesion Molecule

CHAPTER 1. Introduction

Innate immunity and Inflammation

Inflammation can be regarded as a physiological process that maintains homeostasis in response to stress, damage or infection of tissues (Medzhitov, 2008). The inflammatory pathway can be considered to be made up of four components; the inducers, the sensors, the mediators and the effectors of inflammation (Medzhitov, 2008). The inflammatory pathway is tightly regulated. If this tight regulation fails inflammatory programs instead of facilitating homeostasis can cause tissue damage and disease.

Inducers and sensors of inflammation

The inducers of inflammation are the signals that initiate a response and can be categorised as endogenous or exogenous in origin. Exogenous signals are divided into two classes, microbial and non-microbial. Microbial inducers include pathogen-associated molecular patterns (PAMPs) and virulence factors, specialised sensors detect the activities of virulence factors. Non-microbial exogenous inducers include allergens, irritants, foreign bodies and toxic compounds (Medzhitov, 2008). Stressed or malfunctioning tissues within the body produce endogenous inducers of inflammation. Desquamation, the release of cells or molecules that under basal conditions are contained within cellular or tissue membranes, alerts inflammatory sensors to tissue damage that results in the release of inflammatory mediators.

Exogenous microbial inducers of inflammation can be divided into two classes. The first class is the PAMPs that are diverse microbial products including lipopolysaccharide (LPS), peptidoglycan, double stranded RNA and single stranded RNA (reviewed in Janeway and Medzhitov, 2002). These PAMPs are sensed by dedicated receptors, pathogen recognition receptors (PRRs) including the Toll-like receptors (TLRs) and Nod (nucleotide-binding oligomerization domain)-like

receptors (NLRs), that are responsible for mobilization and activation of immune cells which respond by attempting to remove the microbial threat and return the invaded tissue to homeostasis (reviewed in Beutler *et al.*, 2003). Toll receptors were first identified in *Drosophila melanogaster* in which they were found to be important for antifungal defence. Subsequently a homologous family of Toll receptors was identified in mammals (Medzhitov *et al.*, 1997). Many TLRs span the membranes of host innate immune cells sensing microbial inducers and initiating downstream signaling that results in the production of many inflammatory mediators including cytokines, chemokines, and prostaglandins (Anderson, 2000, Beutler *et al.*, 2003, Medzhitov, 2008). The NLRs are intracellular receptors that also recognise bacterial PAMPs. While the TLRs were initially identified in *Drosophila* plants were the model system in which cytosolic sensing proteins were first identified (Philpott *et al.*, 2000, Chisholm *et al.*, 2006). These discoveries later led to the identification of the NLRs including the Nod1 and Nod2 proteins (Girardin *et al.*, 2001, Chamaillard *et al.*, 2003). The second class of exogenous microbial inducer is the virulence factors that are derived from pathogens but not sensed by dedicated receptors. In the case of virulence factors it is the specific effects of the pathogen invasion on host tissues that result in the activation of the inflammatory response. For example pore forming exotoxins produced by bacteria result in the release of K⁺ ions that are sensed by the NALP3 inflammosome (Mariathasan *et al.*, 2006).

Mediators of inflammation

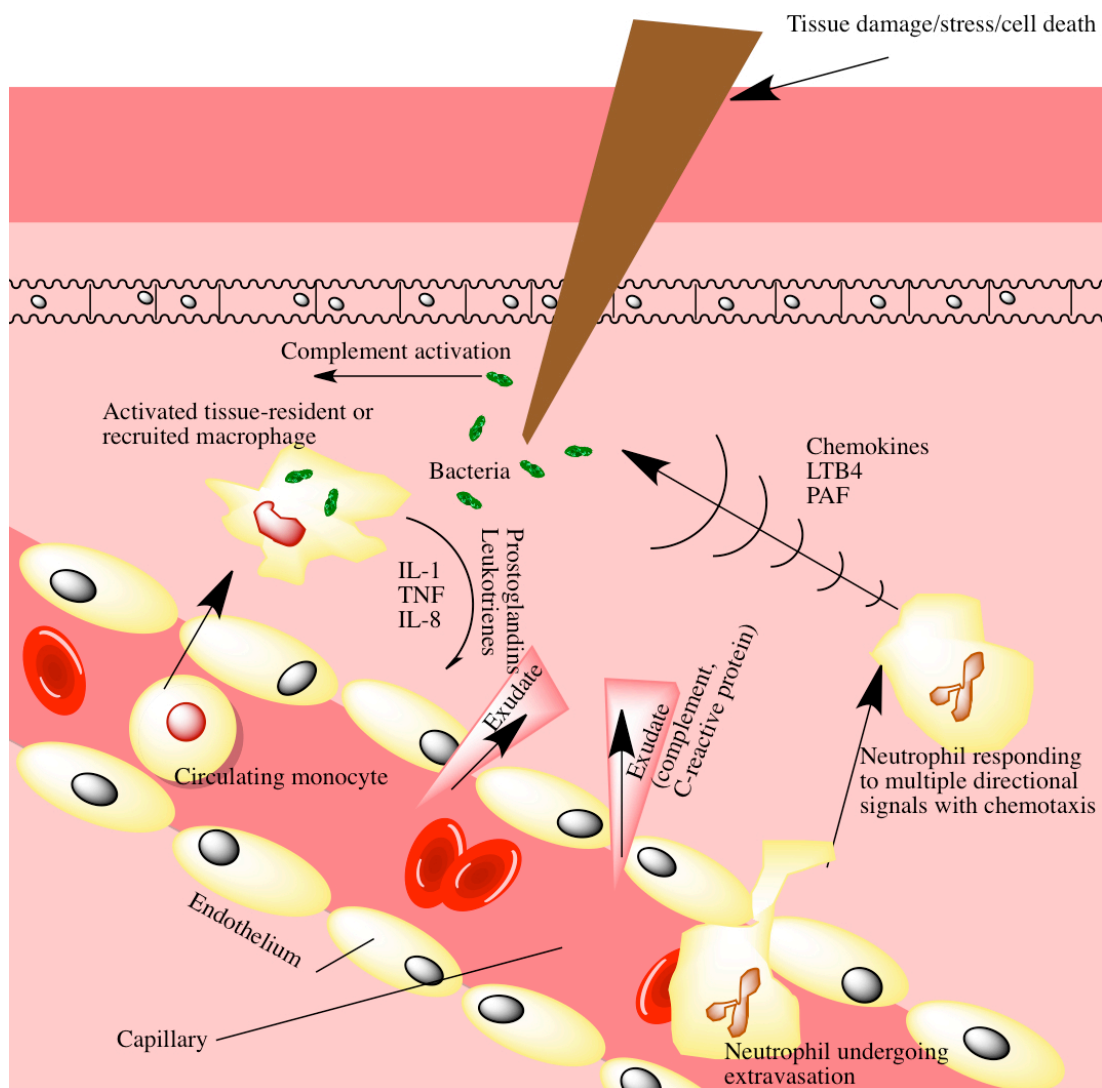
The mediators of inflammation include vasoactive molecules, complement fragments, lipid mediators, inflammatory cytokines, chemokines and proteolytic enzymes (Edvinsson and Owman, 1975, Medzhitov, 2008). Mast cells and platelets release vasoactive amines including histamine and serotonin when they degranulate (Hersh and Bodey, 1970, Mekori and Metcalfe, 2000). These vasoactive amines have dilatory effects on blood vessels close to the site of inflammation and facilitate leukocyte recruitment (Edvinsson and Owman, 1975, Medzhitov, 2008).

Lipid mediators of inflammation, including eicosanoids and platelet-activating factors, have important roles in facilitating leukocyte activation and

recruitment (Kroegel *et al.*, 1989). They are derived from the breakdown of membrane phospholipids. Phospholipids are processed by phospholipase, activated by free Ca^{2+} ions, to produce arachidonic acid and lysophosphatidic acid, which are the precursors for the two classes of lipid mediator, the eicosanoids and platelet-activating factors. Arachidonic acid is processed via the cyclooxygenase (COX1 and COX2) or lipoxygenase pathways to produce prostaglandins and leukotrienes respectively. Prostaglandin production induces vasodilation whilst leukotrienes have a vasodilatory effect and a chemotactic effect on migrating leukocytes (Needleman *et al.*, 1986). Cytokines, including IL-1, $\text{TNF}\alpha$ and IL-6, are produced by many cell types, most importantly macrophages, and mediate many inflammatory functions including activation of the endothelium and leukocytes (Mantovani *et al.*, 1992, Cavaillon, 1994). Chemokines mediate the extravasation and chemotactic recruitment of leukocytes to sites of inflammation (Webb *et al.*, 1993, Tanaka *et al.*, 1993, Medzhitov, 2008).

The classic leukocyte recruitment in response to tissue infection/injury/stress

The immediate immunological response to tissue injury or infection is termed acute inflammation and as the most extreme manifestation of inflammation is the most well understood. Below is a schematic (Schematic 1) that illustrated the classic leukocyte recruitment response to tissue infection/injury/stress.



Schematic 1. Schematic illustration of chemotactic recruitment of macrophages and neutrophils to a site of inflammation. Chemoattractants are released by stromal cells, and inflammatory targets (bacteria or dying cells). Schematic produced referencing Witko-Sarsat *et al.*, 2000 and Kindt *et al.*, 2007.

Effectors of inflammation - leukocyte migration and function in inflammation

Effectors of inflammation are the tissues and cells that respond to the mediators of inflammation as described below for the response to injury and infection.

Innate immune cell types involved in inflammation in response to injury and infection

Classically innate immune cell lineages have been defined by the combination of phenotype, namely cell surface markers and functional criteria. This method of classifying cell types has been challenged in recent years and it is now thought that the number of innate immune cell types we can define is only limited by the number of surface markers we use to classify them (Hume, 2006). Leukocytes show remarkable phenotypic and functional heterogeneity dependent on the different tissues they occupy (Gordon and Taylor, 2005) and plasticity in that changes in the microenvironment can modulate their phenotype. For example, in the presence of TLR mediated signalling and TGF β receptor I blockade type 2 (M2) macrophages are re-programmed to a type 1 (M1) phenotype. Grouping cells involved in the immune response by function and cell surface phenotype has greatly increased our understanding of the biology of the immune response to injury and infection. Until we have much greater understanding of the complex inter and intra cellular networks that make up the immune system it is necessary entertain the concept of innate immune cell lineages.

Neutrophils

The main functions of neutrophils at sites of inflammation are phagocytosis (Brewer, 1963), degranulation (Hirsch and Cohn, 1960, Hirsch, 1962) and killing of microorganisms (Brewer, 1972, Witko-Sarsat *et al.*, 2000). They also mediate the

recruitment, activation and programming of antigen presenting cells (APCs) (Chertov *et al.*, 1997, Bennouna *et al.*, 2003, Nathan, 2006) and drive proliferation of T and B cells (Scapini *et al.*, 2005, Ethuin *et al.*, 2004). Although neutrophils secrete much lower levels of cytokines than mononuclear phagocytes they are often present in numbers orders of magnitude higher than mononuclear phagocytes and so are important producers of activating cytokines (Nathan, 2006). Whilst macrophages can be tissue resident, namely they are present in tissues that are in homeostasis, neutrophils will only exit the blood stream and enter tissues that are stressed.

Neutrophil recruitment to sites of inflammation

Neutrophils have both anti-infectious and pro-inflammatory roles and are the first leukocytes to cross the endothelium and migrate to the site of inflammation. The rapid response of neutrophils to inflammatory stimuli requires the ability to migrate rapidly across the endothelium and move from circulating blood cells to specific sites of inflammation. This rapid response is initiated by the appearance of new adhesion molecules on the endothelium closest to the inflamed site. These adhesive molecules cause the neutrophil to associate with the endothelium and in combination with the shear force produced by the blood flow results in “rolling” of the neutrophil along the endothelium (Atherton and Born, 1972). The first of these adhesion molecules to be identified was L-selectin (leukocyte-selectin, expressed on neutrophils, monocytes and lymphocytes) (Gallatin *et al.*, 1983, Bevilacqua and Nelson, 1993), and subsequently P-selectin (platelet-selectin, expressed on platelets and endothelial cells) and E-selectin (endothelial-selectin, expressed on endothelial cells) were identified (Hsu-Lin *et al.*, 1984, Bevilacqua *et al.*, 1987). L-selectin binds to ligands on some epithelial cells and E and P-selectins bind to ligands on most leukocytes (McEver and Cummings, 1997). The lectin domains of selectins interact with specific glycoconjugate ligands, mainly mucins, and these interactions mediate the rolling of the neutrophil along the endothelium (McEver *et al.*, 1995). Integrins of the $\beta 2$ sub-family are expressed on neutrophils and interact with Inter-Cellular Adhesion Molecule 1 (ICAM-1) molecules on the endothelial cells; these interactions are stronger than those of lectins and their ligands (Sligh *et al.*, 1993,

Muller, 2003). These Integrin/ICAM-1 adhesion interactions eventually become strong enough so that the neutrophil ceases to roll along the endothelium and adheres completely to the surface, this is called arrest or adhesion. Neutrophils require activation by agonists including, platelet-activating factor (PAF), interleukin 8 (IL-8), Formyl-Methionyl-Leucyl-Phenylalanine (fMLP), tumour necrosis factor alpha (TNF α) and Complement component 5a (C5a), before β 2 integrins are able to interact with their ligands (Witko-Sarsat *et al.*, 2000). Cytokine activated endothelial cells can transport neutrophil activating chemokines, for example IL-8, from their abluminal surfaces and by internalisation and transcytose the chemokines to the luminal surface (Middleton *et al.*, 1997).

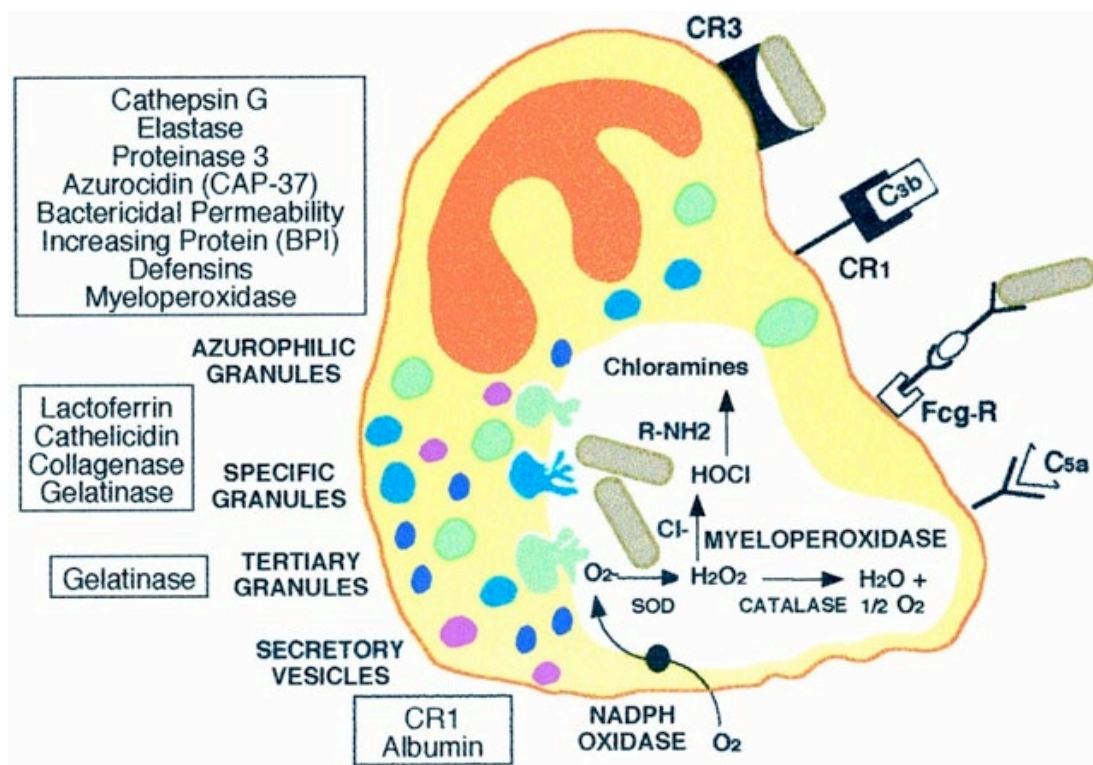
Transmigration of neutrophils across the endothelium can occur via the para-cellular or trans-cellular route. Para-cellular transmigration is the migration of leukocytes from the luminal side of capillaries through endothelial-cell junctions to the abluminal side. Endothelial transmembrane junctional molecules, for example PECAM-1, participate in para-cellular transmigration (Newman, 1997, Vestweber, 2002). Until recently it was thought that para-cellular transmigration was the sole mechanism of neutrophil endothelial extravasation. However trans-cellular neutrophilic migration has been shown to occur (Feng *et al.*, 1998) and whilst it is the route of extravasation for only a minority of cells (Carman and Springer, 2004) it can be very rapid (Cinamon *et al.*, 2004).

Neutrophils as effectors of inflammation

Like macrophages neutrophils are phagocytes. Two classes of receptor expressed on the neutrophil cell surface are responsible for initiation of phagocytosis. These are the Fc γ receptors and the complement receptors. The downstream signalling processes that mediate phagocytosis are different for each class of receptor. Fc γ receptor promoted phagocytosis involves activation of PI3-kinase and Rho proteins and subsequently the formation of the phagocytic cup that eventually engulfs the target particle (Caron and Hall, 1998, Witko-Sarsat *et al.*, 2000). PI3-kinase is required for the final myosin-induced closure of phagosomes to complete

engulfment of particles (Swanson *et al.*, 1999). There is cooperation between complement receptors, CR1 and CR3, and Fc γ receptors as demonstrated by neutrophils from CR3 deficient patients which have impaired antibody dependent phagocytosis (Dana *et al.*, 1984).

Neutrophils contain granules that enclose anti-microbial molecules that are released when neutrophils come into contact with pathogens, this process is called degranulation (e.g. Spitznagel, 1990, Elsbach, 1998, Lehrer and Ganz, 1999, Nathan, 2006). Enzymes within neutrophil granules, including NADPH-dependent oxidase and MPO, contribute to the production of anti-microbial molecules such as reactive oxygen species (ROS) and hydrogen peroxide (H₂O₂). As well as anti-microbial small molecules neutrophil granules contain many anti-microbial peptides and proteins reviewed by Lehrer and Ganz (1999). Neutrophils, once thought to be relatively transcriptionally inactive, have recently been shown to be important producers of inflammatory cytokines (Witko-Sarsat *et al.*, 2000, Nathan, 2006). The anti-microbial molecules released from neutrophil granules can be indiscriminately damaging to host tissue as well as microorganisms (Henson and Johnston, 1987). The release of these non-specific molecules on degranulation by neutrophils can result in tissue damage and ultimately disease (Weiss, 1989). Below is a schematic that illustrates the types of granules found within neutrophils and the anti-microbial/tissue damaging molecules and proteins that they contain (Schematic 2).



Schematic 2. Neutrophil effector mechanisms involved in the defence against pathogens and in the inflammatory process. Neutrophil effector systems are mobilized following phagocytosis of a pathogen. Complement opsonins C3b and C4b are recognized by CR1 and CR3. IgG opsonins are recognized via the immunoglobulin receptors (FcγR). The first microbicidal pathway is the oxidative response, which complex activation, including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and, via myeloperoxidase, hypochlorous acid (HOCl) and chloramines. The second microbicidal pathway is non-oxygen-dependent and consists of the release in the phagolysosome or in the extracellular medium of preformed proteins stored in granules. Serine proteases, antibiotic proteins, as well as myeloperoxidase are contained in azurophilic granules. Metalloproteinases (collagenase and gelatinase) and antimicrobial proteins (lactoferrin and cathelicidin) are contained in specific granules. Gelatinase is also contained in tertiary granules, also called gelatinase granules. This figure and legend are from Witko-Sarsat (2000)

Macrophages

The classical definition of a mononuclear phagocyte is a cell that has stellate morphology, displays evidence of endocytosis, expresses certain enzymes detectable by histochemical staining (e.g. lysosomal hydrolases) and performs non-specific and

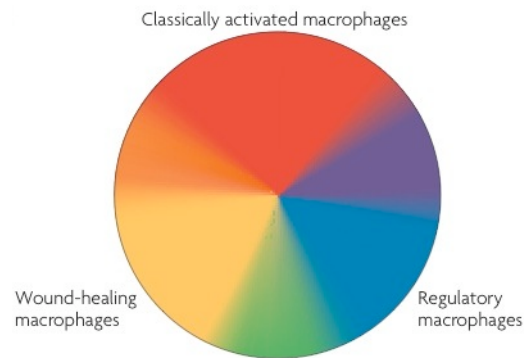
specific uptake of particles (Hume, 2006). Macrophages make up a large component of the mononuclear phagocyte system. They are derived from progenitors in the bone marrow which develop into circulating monocytes that do not proliferate during steady state conditions but have the capacity to enter tissues following inflammatory stimuli and mature into macrophages (van Furth and Cohn, 1968). Whilst monocytes respond to signals and migrate to sites of inflammation, tissue resident macrophages are present throughout the tissues of organs in times of homeostasis and sense changes in homeostatic processes (Laskin *et al.*, 2001). The propensity for a monocyte to migrate towards inflammatory lesions, or to enter normal tissues, can be predicted by the heterogenic expression of certain cell markers. Geissman *et al.* identified combinations of mouse monocyte surface markers that corresponded to heterologous surface markers on human monocytes, and these findings indicated it would be possible to study the *in vivo* relevance of monocyte heterogeneity (Geissmann *et al.*, 2003). The large number of stimuli reported to activate macrophages, and the variation in transcriptional responses of macrophages to additive/combinatorial stimuli demonstrates the phenotypic plasticity of this cell type (Hume *et al.*, 2002). The phenotypic plasticity of macrophages means they can adopt different functional roles within the tissue depending on the environmental stimuli they experience (Gordon and Taylor, 2005).

Macrophages as effectors of inflammation

Monocytes are recruited to sites of inflammation from the blood stream in a similar way to neutrophils. Neutrophils are the first leukocyte to migrate across the epithelium in response to inflammation and are active in recruiting other cell types including monocytes that, after several hours, cross the endothelium and enter the inflamed tissue where they differentiate into inflammatory macrophages. This later recruitment of monocytes is regulated by expression of monocyte selective adhesion molecules by the endothelium, for example Vascular Cell Adhesion Molecule-1 (VCAM-1), which is expressed later compared to the neutrophil adhesion molecules (Chan *et al.*, 2001).

At the site of inflammation macrophages are directed by the mediators of inflammation and respond to varied environmental signals by changing phenotype and function. In some circumstances environmental signals will result in enhanced antimicrobial activity and increased immune function. However some environmental cues can result in macrophages that are more susceptible to pathogenic infections and less able to produce inflammatory cytokines (Mosser and Edwards, 2008). Macrophages are responsible for the clearance of general cellular debris produced when tissues undergo remodelling, and the phagocytosis of cells that have undergone apoptosis. In general these tasks do not activate the macrophage and do not induce the production of inflammatory mediators, in fact the macrophage's principal role can be considered to be that of house keeping by maintaining homeostasis within tissues, which it carries out independent of other immune cells (Mosser and Edwards, 2008). However tissue damage that results in the cell death by necrosis rather than apoptosis will activate the macrophages that clear the cell debris. For example the HMGB1 (high-mobility group box 1) is a protein usually restricted to the nucleus, however on necrotic cell death it is released into the extracellular matrix and acts as an endogenous mediator of inflammation and tissue damage (Tsung *et al.*, 2005, Zhang and Mosser, 2008). Endogenous inflammatory signals activate the macrophage that then produces cytokines and other mediators of inflammation. The presence of microbes and their products and components will also activate the macrophage and induce the production of inflammatory mediators. The plasticity of macrophage phenotype and behaviour has resulted in attempts to classify the different types of activated macrophages based on the activating stimulus and the resulting effector function (Gordon, 2003, Mosser and Edwards, 2008). Macrophages were classed as M1 (classically activated) or M2 (alternatively activated) however this classification has recently been challenged and a new classification of macrophage activation based on three different homeostatic activities proposed (Mosser and Edwards, 2008). The three homeostatic functional groups proposed are: host defence (classically activated), wound healing and immune regulation (Schematic 3). Furthermore the three macrophage groups based on homeostatic functions are described as flexible with sliding scales of classification as

macrophages can evolve to share the characteristics of more than one group (Schematic 3).



Schematic 3. The three populations of macrophages (proposed by Mosser and Edwards 2008) are arranged according to the three primary colours, with red designating classically activated macrophages, yellow designating wound-healing macrophages and blue designating regulatory macrophages. Secondary colours, such as green, may represent tumour-associated macrophages, which have many characteristics of regulatory macrophages but also share some characteristics of wound-healing macrophages. In obese individuals, wound-healing macrophages may transit towards a classically activated-macrophage phenotype. Adapted from Mosser and Edwards 2008.

Classically activated macrophages are defined as the effector macrophages that are produced during cell mediated immunity and are characterised by their activation by the inflammatory mediators $\text{IFN}\gamma$ and $\text{TNF}\alpha$ resulting in their enhanced microbicidal capacity and inflammatory cytokine production (Mackaness, 1977, Gordon and Taylor, 2005, Mosser and Edwards, 2008). Classically activated macrophages are required for efficient host defence of intracellular pathogens. Classical activation of macrophages can occur in response to innate and adaptive signals and results in complex intracellular signal transduction that activates transcription factors including $\text{NF-}\kappa\text{B}$ and the MAPKs (O'Shea and Murray, 2008). Defects in the $\text{IFN}\gamma$ signalling pathway results in increased susceptibility to bacterial and viral infections (Filipe-Santos *et al.*, 2006). However the inflammatory mediators produced by classically activated macrophages are damaging to tissues and can result in immunopathology, for example rheumatoid arthritis (RA) (Szekanecz and Koch, 2007).

Tissue-resident macrophages are often the first members of the immune system to be alerted to tissue stress or infection. Almost any noxious signal, or

deviation from homeostasis, within tissues will alert these tissue-resident macrophages and result in recruitment of first neutrophils and later more macrophages (Hume *et al.*, 2002). Wound healing macrophages are tissue resident macrophages that are activated by IL-4, one of the first inflammatory mediators to be produced after tissue injury (Loke *et al.*, 2007). IL-4 stimulates tissue resident macrophages to produce precursors of the components of the extracellular matrix and begin to contribute to tissue repair and healing (Gratchev *et al.*, 2001, Kreider *et al.*, 2007). The primary source of IL-4 is thought to be from immune cells of the adaptive immune system (Mosser and Edwards, 2008).

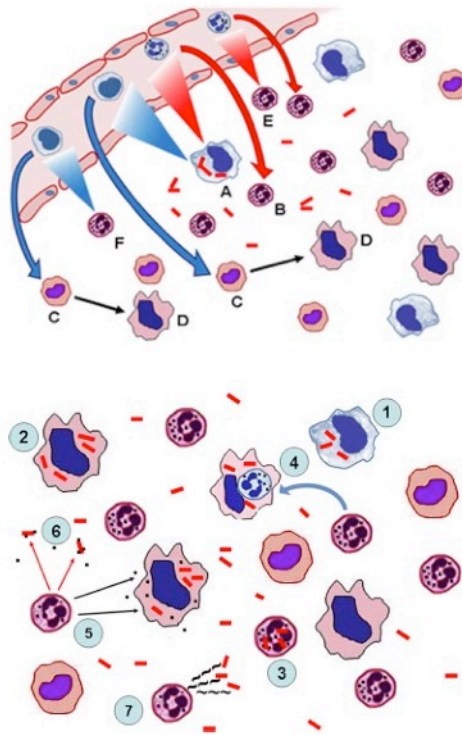
Like classically activated and wound-healing macrophages regulatory macrophages can be activated by innate or adaptive responses. Glucocorticoids released during stress responses have an inhibitory effect on macrophage inflammatory behaviour and result in a reduction in the production of inflammatory mediators and the generation of the regulatory macrophage phenotype (Sternberg, 2006, Mosser and Edwards, 2008). Macrophage phagocytosis is not inhibited by glucocorticoid stimulation (Liu *et al.*, 1999) in fact phagocytosis of apoptotic cells results in the production of TGF β which can contribute to the immunoregulatory function of macrophages (Fadok *et al.*, 1998). The production of the anti-inflammatory cytokine IL-10 is a characteristic of regulatory macrophages (Edwards *et al.*, 2006). There is a requirement for two stimuli in order to generate regulatory macrophages. The first stimulus, for example ingestion of apoptotic neutrophils, prostaglandin stimulation or adenosine (Mosser and Edwards, 2008), combines with a second inflammatory stimulus, for example TLR signalling, resulting in the production of the most important characteristic of regulatory macrophages, the production of IL-10 (Edwards *et al.*, 2006). Regulatory macrophages differ from wound healing macrophages in that they do not contribute to the production of extracellular matrix and they have higher levels of co-stimulatory molecules, for example CD80 and CD86, such that they can function as antigen presenting cells in T cell activation (Edwards *et al.*, 2006, Mosser and Edwards, 2008). The plasticity of macrophages allow them to contribute to tissue remodelling, wound healing and host defence demonstrating the unequivocal requirement for macrophages to maintain homeostasis.

Dendritic cells

There is controversy over the whether dendritic cells (DCs) constitute their own immune cell lineage or are a subset of the macrophage lineage separate from the mononuclear phagocyte system (e.g. Hume, 2006, Geissmann *et al.*, 2010). This debate is ongoing and recently DCs were redefined as a subset of the macrophage lineage and have the primary role of antigen presentation and regulate the pathogen specific adaptive immune response (Hume, 2008). They were first described in the 1970s as a large stellate cell with properties distinct from mononuclear phagocyte cell types (Steinman and Cohn, 1973). DCs process proteins to produce peptides that are presented to T cells via major histocompatibility complexes (MHC) molecules (Itano and Jenkins, 2003). The surface markers that are used to distinguish macrophages from DCs in the lymphoid organs are expressed differently by macrophages and DCs when in other tissues (reviewed in Geissmann *et al.*, 2010). Currently there is no specific single cell surface marker that designates a cell as a DC however they can be defined functionally by their ability to localize to the T cell zone of lymphoid organs where they stimulate T cells (Geissmann *et al.*, 2010).

Macrophages and neutrophils work in concert to combat microbial infection

It is commonly thought that macrophages operate against intracellular pathogens whilst neutrophils operate against extracellular pathogens. Macrophages and neutrophils share a common origin and as such share functionalities including phagocytosis, kinetic behaviour, anti-microbial and immunomodulatory activity (reviewed in Silva, 2009). Macrophages and neutrophils work in concert at the site of infection to present a combined inflammatory response that attacks the invading pathogen and restores tissue homeostasis. Silva presented a comprehensive review of the data that supports macrophage/neutrophil collaboration in acute inflammation and this cooperation is summarised here in the schematic taken from his review (Schematic 4, (Silva, 2009)).



Schematic 4. Schematic representation of the clustering and interactions of neutrophils, monocytes, and macrophages at inflammatory sites of infection by extracellular or intracellular pathogens. (Upper panel) Resident macrophages that detect the presence of invading pathogens (red rods) phagocytose them (A) and secrete chemokines, primarily recruiting neutrophils (such as CXCL8 and CXCL1/2/3; large red gradient) and monocytes (such as CCL2 and CCL3/4; large blue gradient). Recruited neutrophils become activated (B). Activated neutrophils complement the macrophage recruiting of inflammatory phagocytes by attracting additional inflammatory neutrophils (E) and monocytes (F) through the secretion of CXCL8 and CXCL1/2/3 (small red gradient) and CCL2 and CCL3/4 (small blue gradient), respectively. Recruited monocytes (C) give rise to inflammatory macrophages (D). (Lower panel) Infectious focus with resident macrophages clustered with recruited inflammatory neutrophils, monocytes, and macrophages (same cell styles as in upper panel). Effector mechanisms activated by phagocytes against invading pathogens (red rods) include: phagocytosis by resident (1) and inflammatory macrophages (2) and by recruited neutrophils (3); infected macrophages ingest neutrophils (4) and released neutrophil granule proteins (5) to enhance their antimicrobial capacity; activated neutrophils release granules and antimicrobial granule proteins that directly kill pathogens free in the extracellular space (6); neutrophil extracellular traps kill pathogens free in the extracellular space (7). This schematic and legend is from Silva (2009).

Mast cells

Mast cells have important roles in innate and adaptive immunity and are important players in allergic inflammation. They sense inducers of inflammation through Fc receptors and can also be activated by complement components. In acute inflammation they release important inflammatory mediators including histamine, proteoglycans, proteases, leukotrienes, prostoglandins, cytokines and chemokines (e.g. Metcalfe *et al.*, 1997, Prussin and Metcalfe, 2003)..

The migration of immune cells through the interstitium

Much progress has been made in advancing our understanding of leukocyte activation and extravasation, however less is understood about the mechanics of leukocyte migration once they leave the blood stream and enter the tissues. After leukocytes go through the vascular basement membrane and enter the extravascular space they migrate along chemotactic gradients through the interstitium towards the site of inflammation. The interstitium is a three-dimensional environment constructed of a fibrillar network or densely packed cells. Leukocytes undergo amoeboid type movement consisting of fast reorganisation of cell shape to facilitate locomotion (Nourshargh *et al.*, 2010). Movement of leukocytes within the interstitium is controlled by intracellular reorganisation of the actomyosin cytoskeleton. Leukocyte actin polymerisation is regulated by Rac proteins. Deletion of the RAC1 and RAC2 proteins together in neutrophils or DCs results in rounded cells with no capacity for migration (Benvenuti *et al.*, 2004, Sun *et al.*, 2004, Nourshargh *et al.*, 2010). The WASP family of proteins are involved in actin remodelling downstream of the Rac proteins (Snapper *et al.*, 2005, Park *et al.*, 2008). Myosin II is responsible for contractile pressures that propel matter forwards within the cell and also for detachment of the trailing edge of the cell to facilitate forward movement (Lammermann *et al.*, 2008, Xu *et al.*, 2003). A better understanding of the mechanisms of extravasation and extravascular migration will be beneficial for the identification of therapeutic molecular targets. For example increased knowledge of

the molecular mechanisms of leukocyte transendothelial and interstitial migration would allow more selective targeting of leukocyte migration in disorders including atherosclerosis, cancer and inflammatory disorders (reviewed in Nourshargh *et al.*, 2010).

Leukocytes in the interstitium respond to chemotactic gradients, which initiate signalling cascades resulting in chemotaxis. The effects of chemoattractants on neutrophil migration are mediated by many G-protein-coupled receptors (GPCRs). Signals including IL-8, C5a and fMLP are mediated by GPCRs and the resulting signalling stimulates the activation of a large number of intracellular signalling effectors including the Rho GTPases, Phosphoinositide 3-kinases (PI3Ks), and phospholipases (e.g. Xu *et al.*, 2003, Stephens *et al.*, 2008). The mechanisms by which the migrating cell senses the gradient of chemoattractant and implements a directional bias are not fully understood. It is now thought that sampling of the chemoattractant concentration occurs at the leading edge of the migrating cell at points of protrusion. One theory suggests that protrusions at the leading edge are created randomly in space and the protrusion that is exposed to the highest concentration of chemoattractant will be maintained over those exposed to lower concentrations resulting in biased movement up the gradient of signal (Arriemerloulou and Meyer, 2005, Stephens *et al.*, 2008).

Leukocyte recruitment to injury in the absence of infection

The mechanisms underlying leukocyte recruitment to injury in the absence of infection, for example injuries induced by crushes and burns, are poorly understood when compared with the depth of knowledge regarding the acute inflammatory response to injury in the presence of infection. An elegant study has recently used models of sterile injury, namely major trauma in the absence of open wounds and damage to intestinal integrity, to show that mitochondrial danger-associated molecular patterns (DAMPs), are released into the circulation following sterile trauma (Zhang *et al.*, 2010a). Mitochondria are endosymbionts originally derived from bacteria and consequently DAMPs bear bacterial molecular motifs. They are released following cell injury and activate human neutrophils through formyl peptide

receptor-1 and TLR9 leading to MAPK and the activation of signalling pathways usually associated with the sensing of bacterial PAMPs (Zhang *et al.*, 2008).

Acute and chronic inflammation and the resolution of inflammation

The mediators of the resolution of inflammation

Resolution of inflammation, once thought to be a passive process, has now been shown to involve active biochemical programmes that return inflamed tissue to homeostasis (Serhan *et al.*, 2008). Resolution of inflammation occurs when neutrophil recruitment to a site of inflammation ceases, macrophages clear apoptotic neutrophils, macrophages depart through the lymphatics and leukocyte cell numbers to return to pre-inflammation levels (Serhan and Savill, 2005). Mediators of the resolution of inflammation have anti-inflammatory and pro-resolutionary properties and include lipid mediators like the eicosanoids (e.g. lipoxins) (Levy *et al.*, 2001), and newly identified chemical lipid mediators, the resolvins and protectins (Serhan *et al.*, 2002). Resolution of inflammation is distinct from anti-inflammatory processes because pro-resolution mediators actively promote clearance of microorganisms and apoptotic cells (Serhan *et al.*, 2008). So mediators of the resolution of inflammation are distinct from immunosuppressors of inflammation as they activate mechanisms that bring about the restoration of homeostasis in inflamed tissue. A few hours after entering the tissues neutrophils switch from releasing arachidonic acid derived prostaglandins and leukotrienes to the pro-resolutionary arachidonic acid derived lipoxins, and so the initiators and terminators of inflammation have common origins (Serhan and Savill, 2005). It has also now been shown that lipid resolution mediators of resolution are derived from omega-3 polyunsaturated fatty acids precursors including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Serhan *et al.*, 2002). Lipoxins are lipoxygenase-derived eicosanoids and mediate the inhibition of neutrophil entry to the tissues via chemotaxis and adhesion, as well as the uptake of apoptotic neutrophils at sites of inflamed tissue by macrophages (Godson *et al.*,

2000). Neutrophils from resolving inflammatory exudates switch from producing pro-inflammatory leukotrienes to resolving molecules like lipoxins and resolvins (Levy *et al.*, 2001). Resolvins were first identified from murine dorsal air pouch exudates in the spontaneous resolution phase (Serhan *et al.*, 2000). The resolution of inflammation is now considered to be a regulated and integral component of the acute inflammatory response and its dysregulation can lead to chronic inflammation and disease.

Chronic inflammation

Failure to resolve acute inflammation and return tissues to homeostasis results in chronic inflammation and disease. Chronic inflammation occurs when there is a failure to resolve inflammation induced by pathogens or endogenous signals and is characterised by the accumulation of activated macrophages. There are many chronic inflammatory disorders which effect many tissue and organs and include chronic obstructive pulmonary disease (COPD), atherosclerosis, psoriasis, inflammatory bowel disease (IBD), and RA. RA is a classic inflammatory disease and I will use this disease to illustrate the characteristics of chronic inflammation. RA is thought to be initiated by an unknown stimulus that instigates local inflammation, although due to its MHC class II association is likely to involve antigen recognition and T cell activation. This local inflammation activates cells in the synovial lining resulting in the recruitment of immune cells to the joint, which under normal conditions would have no immune cells. In RA leukocytes including neutrophils, macrophages, B-cells and T-cells are present in the joint (e.g. Wong and Lord, 2004). Cytokines including TNF α and IFN γ contribute to the immune processes that result in the pathology of chronic inflammatory conditions including RA. TNF α is crucial to the pathology of RA and it is now the standard target in the treatment of patients with RA (McInnes and Schett, 2007). Anti-TNF α antibodies and IL-1 receptor agonists are strategies for the treatment of RA. However whilst these treatments do ameliorate the symptoms of RA they do not eliminate the disease (Wong and Lord, 2004). B cells produce autoreactive antibodies and contribute to the

recruitment of other leukocytes including neutrophils (Silverman and Carson, 2003). T cells are proposed as the drivers of inflammation in RA by cellular interactions, cytokine production and their interaction with and activation of macrophages (e.g. Brennan *et al.*, 2002, Wong and Lord, 2004). Macrophages in the synovial membrane are activated demonstrated by their production of cytokines and over expression of MHC molecules and the number of macrophages strongly correlates with joint damage in patients with RA (Kinne *et al.*, 2000, Wong and Lord, 2004). Neutrophils are found in an activated state the synovial fluid of RA patients where they produce IL-8, complement C5a and leukotriene B4 (Lopez *et al.*, 1995, Wong and Lord, 2004). Neutrophil apoptosis is inhibited in the synovial fluid and this results in increases in joint damaging neutrophil derived proteinases, hydrolases and ROS (Wong and Lord, 2004). The complex nature of the factors involved in disease pathogenesis in RA and the absence of the understanding of the underlying causes of RA mean that, whilst treatments are available that can relieve the symptoms, so far no cure has been found.

Fibrosis

Chronic inflammation arising from pathogenic or physiological induced tissue damage can result in fibrosis which can occur in many different organs, including lung, kidney and liver, and results in pathology (Cohen, 1995, Ward and Hunninghake, 1998, Kershenovich Stalnikowitz and Weissbrod, 2003). Fibrotic pathology is characterised by activation of fibroblasts and the accumulation of collagenous extracellular matrix (ECM) (Raghow, 1994, Johnson, 1996, Nakatsuji *et al.*, 1998, Sivakumar and Das, 2008). Fibrotic diseases are difficult to treat and patients have poor prognosis and survival (Noble, 2006, Sivakumar and Das, 2008).

Para-inflammation

Recently the concept of para-inflammation has been introduced and occurs in tissues that have not incurred any direct injury or infection but rather a malfunction

or stress to the tissue requires physiological action in order to restore the tissue to a homeostatic state. The tissue -resident macrophages are the first cells that sense the stress or malfunction and if the changes are considerable they recruit additional macrophages and other leukocyte cell types. This inflammatory response in the absence of injury or infection is termed para-inflammation as it is considered a state between the basal and traditional inflammatory state. If inflammatory responses do not return the stressed/malfunctioning tissue to homeostasis para-inflammation can become chronic inflammation (Medzhitov, 2008).

The role of the adaptive immunity in resolution of inflammation and infection

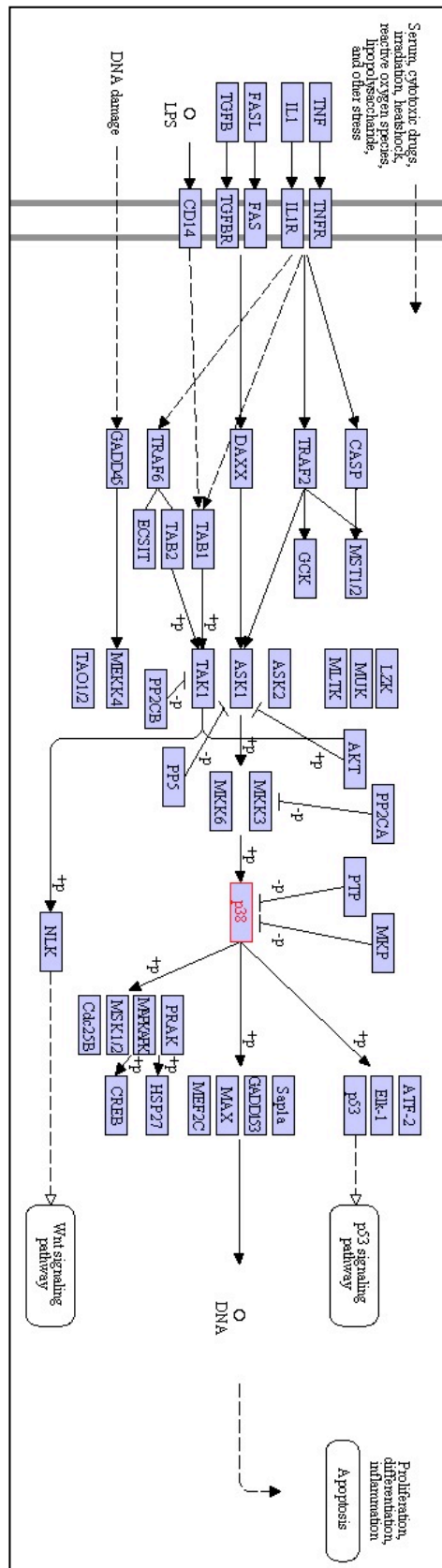
The main focus of this literature review has been to outline the nature of inflammation in the context of innate immunity as this component of the host response relates directly the scientific aims of my thesis project. However adaptive immunity also plays a central role in restoring tissue homeostasis following inflammation. The innate immune system can be defined as “all aspects of the host's immune defence mechanisms that are encoded in their mature functional forms by the germline genes of the host” (Chaplin, 2010). The adaptive immune response involves tightly regulated interactions between APCs and T and B cells that “facilitates pathogen-specific immunologic effector pathways, generation of immunologic memory, and regulation of host immune homeostasis” (Bonilla and Oettgen, 2010). Where the innate immune system recognises pre-programmed patterns the molecules of the adaptive immune system are more specialised and recognise specific antigens and epitopes. The adaptive immune system has evolved in response to the ability of pathogens to mutate and avoid host detection (Pancer and Cooper, 2006). Through a process called somatic recombination, the rearrangement of the immunoglobulin V, D and J gene fragments, receptors are custom made and this diversity means that there is no limit to the number of pathogen components the immune system can recognise (reviewed in Bonilla and Oettgen, 2010). In addition to antigen specificity and altered kinetics of response, namely primary versus secondary immune responses, memory is a key feature which distinguishes adaptive

and innate immunity. Reviewing the contribution of adaptive immune responses in inflammation is beyond the scope of this study which focuses on the role of the innate immune system in inflammatory processes.

p38 MAPK as a mediator of inflammation

MAPKs belong to a family of highly conserved serine/threonine protein kinases of which there are three main classes, the extracellular signal-regulated kinases (ERKs), the c-jun N-terminal kinase (JNK) and the p38 MAPKs. The signalling cascade that activates all MAPK is conserved from yeast to mammals (Johnson and Lapadat, 2002). Of the three main classes of MAPK p38 is considered to be the most promising therapeutic target for inflammatory diseases.

p38 MAPK has multiple roles in the intracellular regulation of many cellular responses to stress stimuli. As well as having important, and well characterised, roles in the regulation of apoptosis, cell-cycle, tumorigenesis and cell differentiation, p38 MAPK plays a central role in the regulation of cellular responses to inflammatory signals and is a key mediator of inflammatory processes. For this reason p38 MAPK has become an important target in the search for therapeutic drugs to treat inflammatory disorders, for example asthma (Adcock *et al.*, 2008), RA (Thalhamer *et al.*, 2008) and atherosclerosis (Osman *et al.*, 2008). p38 MAPK is an intracellular component of complex inflammatory signal transduction pathways (Lee and Young, 1996). The basic p38 MAPK signal transduction pathway is illustrated in Schematic 5.



Schematic 5. Schematic of the p38 MAPK signal transduction network produced by Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa *et al.*, 2010)

p38 MAPK regulates the production of important inflammatory mediators and was first identified as a MAP kinase which could be targeted by LPS and hyperosmolarity (Han *et al.*, 1994). Later that year p38 MAPK was identified again in a pharmacological screen for compounds that modulate the production of inflammatory mediator TNF α (Lee *et al.*, 1994). Four isoforms of p38 MAPK have been identified (α , β , γ & δ) which each have more than 60% sequence homology overall however have differences in their tissue expression, upstream activators and downstream targets (Coulthard *et al.*, 2009). The p38 MAPK subfamily can be divided into two subsets, one subset p38 α and p38 β and the other p38 γ & p38 δ . These subsets are based on their amino-acid sequence identity and their capacity to be inhibited by low concentrations of the inhibitor SB203580. p38 α and p38 β are inhibited at low concentration of SB203580 whilst p38 γ & p38 δ are not (Kumar *et al.*, 1997). SB203580 inhibits ATP binding in the ATP binding pocket when a fluorine atom present on the drug interacts with Thr106 in the hinge of the binding pocket and the subsequent orientation of the drug in the pocket prevents ATP binding (Gum *et al.*, 1998). The duration and magnitude of the activation of p38 MAPK is transient. Activation occurs within minutes of the stimulation signal and is quickly downregulated by protein phosphatases. These include PP2C (Ser/Thr phosphatase) and PTP (Tyr phosphatase) (Takekawa *et al.*, 1998, Takekawa *et al.*, 2000). The M3/6 and MKP phosphatases have also been shown to regulate p38 MAPK activation (Keyse, 2000, Tanoue and Nishida, 2003, Liu *et al.*, 2007).

Inhibitors of p38 MAPK block the production of TNF α , IL-1, IL-8 and COX2 by monocytes (Lee *et al.*, 1994, Westra *et al.*, 2004). p38 can also regulate TNF α and IL-1 at the transcriptional and translational levels (Lee *et al.*, 1994, Campbell *et al.*, 2004, Baldassare *et al.*, 1999). In mice that lack the p38 direct downstream target MAPK-activated protein kinase 2 (MAPKAP-K2) there is a defect in TNF α and IL-6 cytokine production on stimulation with LPS (Kotlyarov *et al.*, 1999). The p38 α isoform is thought to be responsible for cytokine production as mice lacking the p38 β isoform, which is the isoform

most closely related to p38 α , have no defects in cytokine production (Beardmore *et al.*, 2005). p38 MAPK regulates post translational gene expression by regulating the stability of mRNAs encoding many inflammatory mediators including TNF α , IL-1, IL-6, COX-2 and many more (Clark *et al.*, 2003). Inflammatory transcriptional targets of p38 MAPK in the context of a rat model of RA are illustrated in Table 1.

Gene Name	Systematic Name	Description	Ratio of TNF- α (+) to TNF- α (-)	Signal Intensity of TNF- α (+)	Ratio of SB80 (+) to SB80(-) (+TNF- α)
Cytokines					
Cxcl2	NM_053647	Chemokine (C-X-C motif) ligand 2	59.83 \pm 27.40	257,302.8	0.46 \pm 0.02
Gm1960	NM_138522	Cytokine-induced neutrophil chemoattractant-2b	3.73 \pm 1.60	298,841.3	0.44 \pm 0.04
Cx3cl1	NM_134455	Chemokine (C-X3-C motif) ligand 1	2.12 \pm 1.00	260,865.1	0.69 \pm 0.09
Il1b	NM_031512	Interleukin 1 β	11.52 \pm 2.85	12,303.8	0.69 \pm 0.39
Bmp2	NM_017178	Bone morphogenetic protein 2	4.50 \pm 1.80	28,567.7	0.75 \pm 0.04
Proteases and protease inhibitors					
Mmp10	NM_133514	Matrix metalloproteinase 10	18.14 \pm 1.91	21,343.8	0.65 \pm 0.14
Mmp3	NM_133523	Matrix metalloproteinase 3	2.53 \pm 1.00	300,288.3	0.56 \pm 0.24
Mmp13	M60616	UMRCase	5.40 \pm 2.12	229,030.9	0.43 \pm 0.05
Serpine2	XM_343604	Serine (or cysteine) proteinase inhibitor, clade E, member 2 (Serpine2)	3.22 \pm 1.20	40,057.2	0.72 \pm 0.12
Metabolism					
Ptges	NM_021583	Prostaglandin E synthase	4.78 \pm 2.37	89,878.9	0.76 \pm 0.12
Ptgs2	NM_017232	Prostaglandin-endoperoxide synthase 2	7.74 \pm 2.78	97,096.8	0.45 \pm 0.05
Pde4b	NM_017031	Phosphodiesterase 4B, cAMP specific	2.01 \pm 0.53	16,247.5	0.67 \pm 0.07
Cell adhesion and motility					
Emp2	NM_001007721	Epithelial membrane protein 2	2.47 \pm 0.23	28,383.0	0.63 \pm 0.16
Arhgdib	NM_001009600	Rho, GDP dissociation inhibitor (GDI) β	3.10 \pm 0.63	22,087.4	0.64 \pm 0.10
Pcdh16	XM_219128	Protocadherin 16 precursor; protocadherin 16	2.17 \pm 0.33	23,333.7	0.58 \pm 0.01
Other genes					
Igf1bp3	NM_012588	Insulin-like growth factor binding protein 3	2.47 \pm 0.52	12,427.3	0.46 \pm 0.09
Igf1bp5	AW917764	Insulin-like growth factor binding protein 5	3.71 \pm 0.47	5,795.1	0.33 \pm 0.02
Ereg	NM_021689	Epiregulin	4.05 \pm 0.79	14,294.2	0.74 \pm 0.43
Tlr2	NM_198769	Toll-like receptor 2	2.59 \pm 0.98	151,051.0	0.61 \pm 0.08
RGD1307189	NM_001008349	Similar to nuclear factor- κ B subunit p100	2.21 \pm 0.19	15,070.3	0.68 \pm 0.13
Slc20a1	NM_031148	Solute carrier family 20 (phosphate transporter)	2.65 \pm 0.91	101,728.4	0.62 \pm 0.07
Pcp4	NM_013002	Purkinje cell protein 4	6.22 \pm 1.57	49,690.0	0.65 \pm 0.03
Tgm2	NM_019386	Transglutaminase 2, C polypeptide	3.62 \pm 2.69	1,668.2	0.46 \pm 0.05

Table 1. Gene expression profiles of rat fibroblast-like synoviocytes upregulated by TNF- α and inhibited by SB-203580. Table taken from Zer *et al.*, 2007.

The role of p38 MAPK as a regulator of inflammatory responses extends to the TLR4 receptor-signalling pathway, where it has been shown to be selectively required for TRAF6-ASK1 dependent TLR4-mediated innate immunity (Matsuzawa *et al.*, 2005). p38 MAPK is also required for phosphorylation and phosphoacetylation of histone H3 selectively occurring on the promoters of cytokine and chemokine genes, enhancing the accessibility of NF- κ B binding sites, in response to LPS and other TLR ligand stimulation (Saccani *et al.*, 2002, Ghosh and Hayden, 2008).

p38 MAPK as well as regulating production of inflammatory mediators regulates the effector function of leukocytes by controlling their migration in response to inflammatory stimuli (Cara *et al.*, 2001, Aomatsu *et al.*, 2008). p38 α , but no other p38 isoform, has been shown to be required for the transduction of chemotactic signals involved in cell migration (Rousseau *et al.*, 2006). N-formyl-L-

leucyl- L-phenylalanine (fMLP) and C5a induced neutrophil chemotaxis requires the relay of chemotactic signals by p38 (Heit *et al.*, 2002, Heuertz *et al.*, 1999).

Inhibitors of p38 MAPK as anti-inflammatory therapeutic drugs

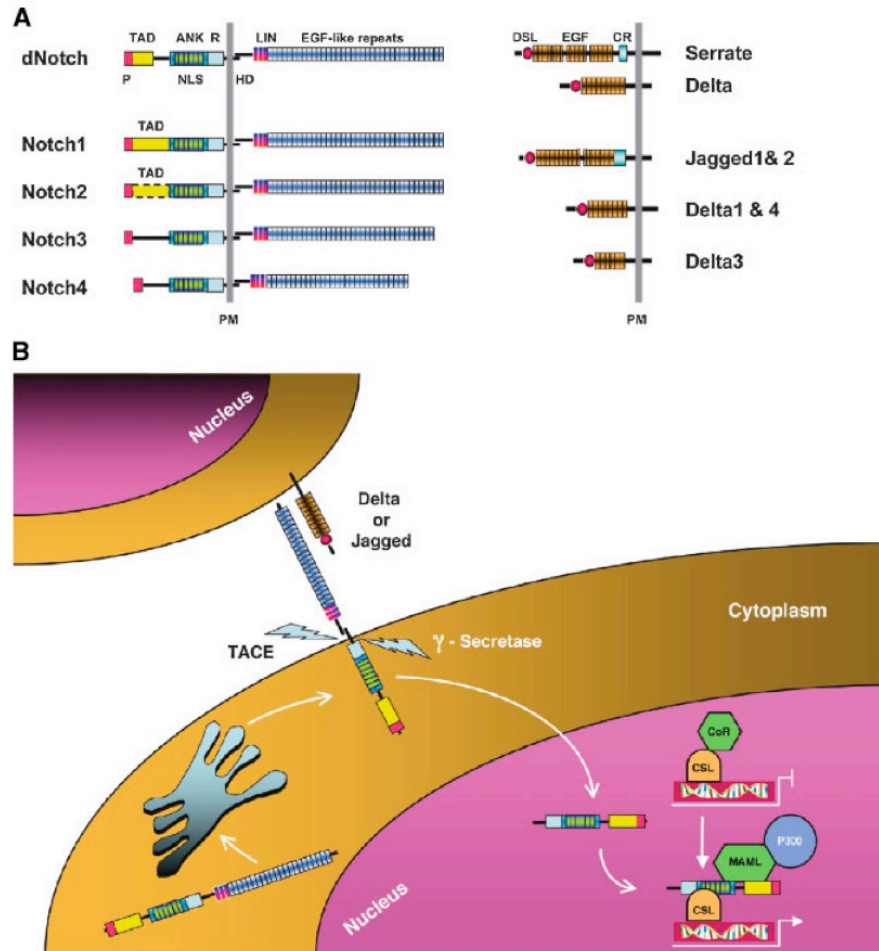
Since inflammatory cytokines are known to cause and exacerbate inflammatory diseases, and p38 MAPK is a key regulator of the production of cytokines, inhibitors of p38 MAPK have been developed in order to test their efficacy as anti-inflammatory therapeutic drugs. Over the last decade there has been great optimism about the potential of p38 MAPK inhibitors as treatments for chronic-inflammatory disorders. However the recently published results of several clinical trials have proven disappointing and it is now considered the optimism surrounding the use of p38 MAPK inhibitors as treatments for inflammatory disorders is over (Goldstein *et al.*, 2010). One inhibitor, AMG 548, reduced TNF α and IL-1 β production by 85% in phase two clinical trials, however production was halted because it caused elevations in liver enzymes (Lee and Dominguez, 2005). Another clinical trial of the highly specific p38 inhibitor pamapimod was shown to be less effective at improving the Disease Activity Score in RA patients than existing treatments (Hill *et al.*, 2008). In a second phase 2 trial testing pamapimod in RA patients the outcome of treatment at the primary efficacy end point was similar in patients that received pamapimod and those that had received the placebo control (Alten *et al.*, 2009). The development of pampimod has been discontinued on the basis of these phase 2 trials. Vertex (VX-745) is another p38 MAPK inhibitor that has been tested in phase 2 clinical trials for the relief of RA symptoms and is the drug that has produced the most encouraging results of any p38 MAPK inhibitor tested in phase 2 trials (Haddad, 2001). Although only limited information was disclosed subsequent reports detailed CNS toxicity in dogs treated with this compound. VX-702 is a modified version of the VX-745 compound that does not cross the blood brain barrier. When tested in clinical trials for the treatment of RA the efficacy of VX-745 was poor with little difference in outcome when compared with the placebo treatment. A second phase 2 study testing VX-745 had similar

results to the first. Neither of these studies demonstrated adverse liver or CNS effects (Damjanov *et al.*, 2009). Another specific and highly potent orally administered small molecule p38 α inhibitor is SCIO-469. This compound is the only p38 MAPK inhibitor that has been tested for analgesic efficacy. TNF α and IL-1 both contribute to and modulate acute and chronic pain. When SCIO-469 was used in trials to treat acute post-surgical dental pain patients that received SCIO-469 showed significantly longer times before they required rescue medication when compared with the patients that had received the placebo (Goldstein *et al.*, 2010). SCIO-469 was also tested in phase 2 trials to treat RA however the response rate between SCIO-469 treated patients and placebo did not reach statistical significance at the primary end point (Goldstein *et al.*, 2010). The p38 inhibitor BIRB 796 was tested in a late stage clinical trial on patients with Crohn's disease. However the outcome of this study revealed that there was no statistical difference in remission rates between BIRB 796 treated and placebo treated patients (Schreiber *et al.*, 2006). ARRY-797 is a p38 inhibitor that has been reported to produce only a transient reduction in CRP in an RA clinical study, which is consistent with the transient reduction of CRP levels seen in other studies. The testing of ARRY-797 in inflammatory diseases has been discontinued and it is suggested that it will continue to be tested for analgesic effects (Goldstein *et al.*, 2010). There are several other p38 inhibitor drugs being tested, mainly for their effect on neuropathic pain, however no clinical results of these studies have been released so far. The ability of p38 inhibition to block the synthesis of inflammatory cytokines resulted in the production of highly specific inhibitors of p38 MAPK. Despite the difficulty in producing specific inhibitors of kinases many highly specific inhibitors of p38 have been produced. Only very recently have the results of phase 2 clinical trials of these compounds been released, and a disappointing pattern of results has emerged. In the four RA trials and one Crohn's disease trial that the results are known for it seems that whilst promising reductions in inflammatory biomarkers were observed early in the studies there was a compensatory mechanism after longer treatment with the p38 inhibitor. It remains to be elucidated whether inhibition of p38 in combination with other therapeutic strategies may yet result in the desired efficacy in treatment of inflammatory diseases (Goldstein *et al.*, 2010).

Notch signalling

Overview of Notch signalling

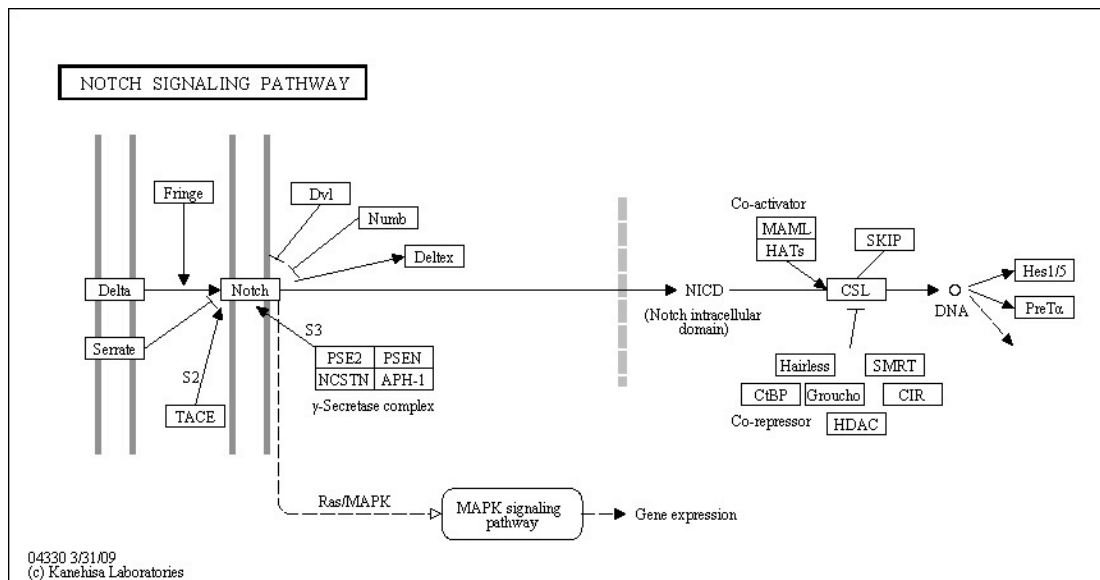
Notch/ligand interactions are important mediators of cell-cell communication and are involved in a wide range of biological processes including growth, survival, differentiation and proliferation (Artavanis-Tsakonas *et al.*, 1999). Notch signalling is highly conserved across species. In 1917 fruit flies with notches in their wings were described (Morgan, 1917). These flies were later found to have loss of function in the *Drosophila* Notch gene, which was cloned in the 1980s (Wharton *et al.*, 1985, Kidd *et al.*, 1986). Mammals have four Notch receptors (Notch 1-4) and five Notch ligands (Delta-like1, 3, and 4 and Jagged 1 and 2) (reviewed in Radtke *et al.*, 2010). A summary of Notch/ligand binding and signalling is outlined in Schematic 6.



Schematic 6. Notch receptors/ligands and signalling. **A:** Notch receptors and ligands. Vertebrates have four (Notch1 – 4). The extracellular domain of the receptors contain between 29 and 36 EGF-like repeats involved in ligand binding, followed by three cysteine-rich Notch/LIN12 (LIN) repeats. The LIN domain is followed by the heterodimerization domain (HD). The cytoplasmic part of the receptor contains two protein – protein interaction domains, the RAM (R) domain, six ankyrin repeats (ANK), two nuclear localization signals (NLS), a transcriptional transactivation domain (TAD) and a PEST sequence. The vertebrate Notch receptors can be activated by at least five ligands (Jagged1 and 2 (homologs of Serrate), and Delta1, 3 and 4 (homologs of Delta)). The common feature of these two ligand families is an N-terminal structure called DSL (Delta, Serrate and Lag). Both type of ligands contain EGF-like repeats in the extracellular domain, but only Serrate, Jagged1 and Jagged2 harbour an additional cysteine- rich (CR) sequence downstream of the EGF-like repeats. PM, plasmamembrane. **B)** Notch signalling. Notch receptors are synthesized as precursor proteins and cleaved in two during transport to the cell surface where they are expressed as heterodimers. Notch signalling is initiated by ligand binding which induces two subsequent proteolytic cleavages, the first mediated by TACE (tumor-necrosis factor α - converting enzyme) near the transmembrane domain, while the second cleavage which occurs within the transmembrane domain is mediated by the γ -secretase activity of presenilins. The liberated cytoplasmic portion of the receptor (NIC) translocates to the nucleus and heterodimerizes with the transcription factor CSL. Binding of NIC to CSL leads to transcriptional activation by displacement of co-repressors (COR) and simultaneous recruitment of coactivators such as Mastermind-like proteins (MAML) that interact with P300. This schematic and legend are from Radtke 2005 (Radtke *et al.*, 2005).

A crucial event in the Notch signalling cascade is the shedding of the ectodomain following ligand binding. This is mediated by proteases of the ADAM/TACE/Kuzbanian family, which regulate cleavage of the receptor resulting in release of the ectodomain (Brou *et al.*, 2000, Mumm *et al.*, 2000, Lieber *et al.*, 2002). The cleavage of the ectodomain results in the production of the membrane bound form of Notch which is called the Notch Extracellular Truncation (NEXT) (Schweisguth, 2004). NEXT is further cleaved at two sites by the activity of intracellular γ -secretase, which is part of the Presenilin- Nicastrin-Aph1-Pen2 protein complex (Fortini, 2001, De Strooper, 2003). The cleavage of NEXT results in the release of the Notch intracellular domain (NICD) into the cytosol (Okochi *et al.*, 2002). Notch signalling can occur via the canonical pathway, CSL dependent (known as CBF1 in humans, RBPJ in mouse and Suppressor of Hairless in flies) dependent, or non-canonical, CSL independent pathway (Martinez Arias *et al.*, 2002). In canonical signalling NICD translocates to the nucleus and forms a nuclear transcription complex with the DNA binding protein CSL and the Mastermind (Mam)/Lag-3 co-activator (known as MAML in humans) (Bray and Furriols, 2001, Petcherski and Kimble, 2000). The formation of this complex results in the transcription of Notch/CSL dependent genes (Schweisguth, 2004).

Notch signalling feeds into the MAPK signalling pathway via the action of Ras, an upstream component of the MAPK signalling pathway illustrated in Schematic 7 (Alberola-Ila and Hernandez-Hoyos, 2003). Notch negatively regulates p38 activation by inducing the expression of MKP-1, a MAPK phosphatase, which directly inactivates p38. This MKP-1 induction is CSL dependent (Kondoh *et al.*, 2007). The degree of cross talk between the intracellular Notch and MAPK pathways is not clear and much work remains to be done to fully elucidate these interactions (Kondoh *et al.*, 2007).



Schematic 7. Schematic of the Notch signalling produced by Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa *et al.*, 2010).

Notch signalling and the immune system

Notch signalling is required for many cell fate decisions during embryogenesis and development. Notch1 and RBPJ are required for the development of embryonic HSCs in mouse (Kumano *et al.*, 2003). Subsequently the role for Notch in HSC development was confirmed when it was shown that Jagged1, but not Jagged2, is required for generation of embryonic HSCs. This same study demonstrated that Jagged1/Notch signalling controlled Runx1 and GATA2 expression, both are important mediators of haematopoiesis (Robert-Moreno *et al.*, 2008). Whilst Notch is required for haematopoiesis in the developing embryo the combined results of several studies indicate that canonical Notch signalling is dispensable for HSC homeostasis in the bone marrow (Radtke *et al.*, 2010).

Notch1 signalling is required for the maturation of T cells (Radtke *et al.*, 1999, Han *et al.*, 2002) However Notch1 does not simply function to instruct a bipotent progenitor cell to become a T cell as recent discoveries have demonstrated that loss of Notch function affects cell fates of myeloid cells and B cells as well as T cell development (Bell and Bhandoola, 2008, Feyerabend *et al.*, 2009, Wada *et al.*, 2008). Notch2/Delta-like 1 (DLL1) signalling is involved in the specification of B cell subsets (Hozumi *et al.*, 2004, Saito *et al.*, 2003). DLL1 stimulation promotes the

development of DCs over other immune cell lineages including T cells and macrophages (Ohishi *et al.*, 2001, Olivier *et al.*, 2006).

Notch signalling extends beyond cell fate decisions in the developing immune system and plays a role in regulating the mature immune system. Notch1 is expressed in CD4+ T cells and the expression of Notch target gene *hes1* is upregulated following activation (Benson *et al.*, 2004). Notch ligand expression is also upregulated on T cells stimulated with inhibitory cytokines (Hoyne *et al.*, 2001). Notch is a co-stimulatory molecule involved in the activation and effector functions of T cells (Benson *et al.*, 2004). Notch is also proposed to have potential implications for the effector activity of B cells (Hoyne *et al.*, 2001). Notch signalling is important for the regulation of T cell viability *in vitro* (Hoyne *et al.*, 2001). It has recently been shown that Notch signalling is required for macrophage M2b polarisation in a murine lupus model via the action of PI3K, ERK and p38 MAPK signalling (Zhang *et al.*, 2010b). Recent evidence also demonstrates the involvement of Notch signalling in the regulation of TLR responses in macrophages including cytokine production, antigen presentation and cytotoxic activity (Monsalve *et al.*, 2006, Hu *et al.*, 2008, Palaga *et al.*, 2008). It has also been implicated in TLR signalling in macrophages through modulation of the IFN γ pathway (Hu *et al.*, 2008). The involvement Notch in the TLR4/LPS recognition pathway will be further discussed in chapter 5.

The use of zebrafish to study inflammation

The zebrafish immune system

In the 1960s George Streisinger set out to find a model organism that was suitable for forward genetic manipulation and appropriate for modelling human diseases. His background as a fish enthusiast led him to choose a small tropical fish, the zebrafish (Grunwald and Eisen, 2002). After more than a decade of experimentation he successfully developed protocols that allowed the identification of germ-line mutations and eventually was able to produce homozygous diploid clones (Streisinger *et al.*, 1981, Meeker and Trede, 2008). A description of zebrafish haematology first entered the literature in 1963 when it was demonstrated that blood cells formed early on in embryo development in the middle somatic region (Colle-

Vandevelde, 1963). Over the following decades zebrafish became a powerful tool in the study of developmental genetics, including the haematopoietic compartment. It is now clear that zebrafish can be used as a haematological model (Carradice and Lieschke, 2008). Zebrafish are genetically tractable and are ideal for large-scale mutagenesis screens. In the 1990s a large scale ENU mutagenesis screen produced over 4000 mutants that displayed defects in development which were used to identify genes involved in the development of the notochord, brain, spinal cord, somites, muscles, heart, circulation, blood, skin, fin, eye, otic vesicle, jaw and branchial arches, pigment pattern, pigment formation, gut, liver, motility and touch response (Haffter *et al.*, 1996). Mutants in haematopoietic genes found by these screens have led to new models for disease and given insights in to developmental haematology (Brownlie *et al.*, 1998). Zebrafish possess both an innate and adaptive immune system. The early immune system of the zebrafish is innate only, providing a tool for elucidating different roles for innate and adaptive immunity in response to inflammation and disease (Lam *et al.*, 2002). The genetic, morphological and functional similarities between the zebrafish and the human immune systems are many (Crowhurst *et al.*, 2002, Lieschke *et al.*, 2001). In fact, zebrafish possess most, if not all, of the cell types of the mammalian immune system (Berman *et al.*, 2003, Traver, 2004). Zebrafish also possess many of the inflammatory mediators associated with the inflammatory response in mammals including orthologues of p38 MAPK, which will be discussed further in chapter four. The suitability of zebrafish as a model to study the mammalian immune system is outlined in Table 1 (taken from a review article by Meeker and Trede (2008)) comparing the zebrafish and mammalian immune system components.

	Human	Zebrafish
General		
Sites of early hematopoiesis	Yolk sac AGM Fetal liver	ICM Dorsal aorta CHT
Site of adult hematopoiesis	Bone marrow	Kidney marrow
Lymphatic system	ZF has no lymph nodes but does have putative lymphatic vessels including a thoracic duct	
Complement system	Well developed in ZF with shared human elements	
Inflammatory proteins	Well conserved between human and ZF, i.e., TNF α , NF- κ B, COX2, IL-1, IL-8 and other C-C and C-X-C chemokines	
MHC	Class I, II, III all present in ZF	
Myeloid		
Neutrophils		
Nuclear lobes	4–5	2–3
Cytoplasm	Heterophilic with azurophilic and non-azurophilic granules similar to human	
Motility	+	+
Phagocytic activity	+	+
Myeloperoxidase	+	+
Acid phosphatase	+	+
Periodic acid Schiff	+	—
Respiratory burst	+	+
Eosinophils		
Morphology	Morphologically distinct from human counterpart	
Monocytes/macrophages		
Motility	+	+
Phagocytic activity	+	+
Respiratory burst	+	+
Ability to activate T/B cells	+	+
Mast cells and basophils	Currently not characterized in ZF	
Lymphoid		
Thymus		
Anatomy	Multilobulated midline structure	Bilateral single-lobed twin structures which remain attached to pharyngeal endoderm
Histology	Demarcated cortex and medulla in human and ZF	
T cells		
Site of initial development	Bone marrow	Kidney marrow
Educated in thymus	+	+
TCR	ZF TCR α , β , δ , γ chains identified	
Rag-dependent V(D)J rearrangement	+	+
Gene expression	Similarities include <i>ikaros</i> , <i>lck</i> , <i>GATA-3</i> , <i>rag</i>	
B cells		
Embryonic development	Initially develop in ZF pancreas	
Ig subtypes	A,D,G,E, M	D, M, Z
Rag-dependent V(D)J rearrangement	+	+
AID	+	+
Class switch recombination	+	—
Somatic hypermutation	+	Inefficient
Antibody response to immunization	+	+
NK cells		
Receptor	Classical NK receptor	Putative NITRs
Abbreviations: AGM = aorta, gonad, and mesonephros; ICM = intermediate cell mass; CHT = caudal hematopoietic tissue; NITR = novel immune-type receptors; ZF = zebrafish.		

Abbreviations: AGM = aorta, gonad, and mesonephros; ICM = intermediate cell mass; CHT = caudal hematopoietic tissue; NITR = novel immune-type receptors; ZF = zebrafish.

Table 2. Overview of the zebrafish immune system and comparison to human. Taken from Meeker and Trede (2008).

The zebrafish has several advantages over its mammalian counterparts as a model animal to study the immune system. Zebrafish is one of the smallest animals with a fully developed innate and adaptive immune system and therefore a large number of laboratory animals can be housed in a small space. These animals are also highly fecund with a single breeding pair able to produce in excess of 200 embryos per week. Additionally the embryos develop *ex-utero* and remain transparent for several days which both facilitate unrivalled visualisation of haematopoiesis and immune cell behaviour in a vertebrate model (Meeker and Trede, 2008). The zebrafish is a genetically tractable model organism. Now that the majority of the zebrafish genome has been fully sequenced, it is currently being assembled at the Sanger Institute, it has become clear that the zebrafish and mammalian genome maps have substantial conservation of syntenies ('a pair of genes is syntenic if they are found on the same chromosome') (Postlethwait *et al.*, 1999). Zebrafish and other teleosts often have multiple copies of a single gene. This is caused by evolutionary genome duplication events (Postlethwait *et al.*, 1998). This gene duplication can present an advantage over mammalian model organisms where knock out of a single gene of interest is embryo lethal. In zebrafish knock down of one copy of a duplicate gene can give insight into the function of that gene without producing an embryo lethal phenotype (Spitsbergen and Kent, 2003). The disadvantages of studying the zebrafish include the shortage of availability of monoclonal antibodies, that zebrafish cells respond poorly to cell culture and that currently there is no conventional gene knockout procedure (Meeker and Trede, 2008).

Zebrafish macrophages develop from the lateral mesoderm and are the first leukocytes to develop and emerge from the yolk sac (Herbomel *et al.*, 1999). By 48 hours post fertilisation (hpf) macrophages and primitive neutrophils have developed and migrated away from the yolk sac (Herbomel *et al.*, 2001, Le Guyader *et al.*, 2008). Once differentiated, some of these early migratory macrophages will move from the yolk sac to enter the circulation, whilst many migrate to the head of the embryo. These embryonic macrophages have several atypical characteristics. They seem to bypass the monocytic stage and retain proliferative capacity once differentiated (Herbomel *et al.*, 1999). The zebrafish orthologue of the *fms* receptor

is known as *csflr* and is expressed alongside the macrophage lineage marker *pu.1* in macrophage progenitors (Parichy *et al.*, 2000). A mutant in the *csflr* gene, the *panther* mutant, was used to demonstrate that *csflr* expression is required in the embryonic macrophage for successful emigration from the yolk sac and pericardial area. Mutations in this gene result in failure of the macrophages to colonise embryonic tissues (Herbomel *et al.*, 2001).

Zebrafish have multi-lobular neutrophils, which highly express myeloperoxidase (MPO) (Bennett *et al.*, 2001). Genes associated with the development of the adaptive immune system are expressed from 48 hpf, however mature lymphocytes do not develop until 3 weeks post fertilisation (Willett *et al.*, 2001). The period between fertilisation and the development of the adaptive immune system gives an opportunity to investigate the responses of the innate immune system to injury or disease in the absence of adaptive responses. Injury models in young embryos allow for screening of genes regulating, and compounds that modulate, leukocyte recruitment. Recently several studies have demonstrated that tail fin injuries such as tail transections (Lieschke *et al.*, 2001, Renshaw *et al.*, 2006, Niethammer *et al.*, 2009) and medial fin incisions (Mathias *et al.*, 2006, Brown *et al.*, 2007) can provide insights into the mechanisms of myeloid cell recruitment to a site of inflammation. Transgenic zebrafish lines exist in which green fluorescent protein (GFP) is expressed under immune cell lineage specific promoters and these will be further discussed in chapter three. The MPO::GFP zebrafish expresses GFP under the neutrophil specific MPO promoter (Renshaw *et al.*, 2006, Mathias *et al.*, 2006). Several studies have demonstrated that the neutrophil mediated inflammation might be resolved by retrograde migration from injured tissue back into the circulation (Mathias *et al.*, 2006, Brown *et al.*, 2007). Whilst the zebrafish embryo is a versatile tool for *in vivo* studies, adult fish can be used to extract blood cells for *in vitro* analysis. In 2003 Travers *et al.* demonstrated that the major blood lineages could be isolated by flow cytometry. The zebrafish blood cell types closely resemble their mammalian counterparts excepting the erythrocytes which are nucleated and the thrombocytes which perform the clotting functions that platelets perform in mammals (Jagadeeswaran *et al.*, 1999).

Zebrafish models of bacterial infection

The similarities between the zebrafish and mammalian immune system have facilitated the emergence of zebrafish embryos and adult models of bacterial infection. Recent studies of Streptococcal infection in zebrafish have contributed to our understanding of bacterial virulence (Neely *et al.*, 2002, Bates *et al.*, 2005, Cho and Caparon, 2005, Miller and Neely, 2005, Montanez *et al.*, 2005, Lowe *et al.*, 2007, Phelps and Neely, 2007, Kizy and Neely, 2009, Lin *et al.*, 2009). *Salmonella* infection experiments in zebrafish have revealed conserved transcriptional responses in fish including TLR signalling, stress response factors and inflammatory mediators (van der Sar *et al.*, 2006, Stockhammer *et al.*, 2009). One of the most successful applications of bacterial infection in zebrafish has been their use in the study of mycobacterial infection. Embryonic infection with *Mycobacterium marinum*, a natural fish pathogen, has allowed the visualisation of infection in real time (Davis *et al.*, 2002b), has shown that the mycobacteria replicate within macrophages and form granulomas as they do in mammalian infections (Clay *et al.*, 2007, Clay *et al.*, 2008), and has demonstrated macrophages in the granuloma undergo cell death and actively recruit macrophages from elsewhere in the embryo (Davis and Ramakrishnan, 2009). Adult zebrafish are also used to study the progression of mycobacterial infection. Adult infections can be chronic when low doses of mycobacteria are administered or acute with higher doses. Experiments using Rag1 fish that have no T-cells or B-cells demonstrated the requirement for adaptive immunity for the control of the infection (Swaim *et al.*, 2006). Different strains of *M. marinum* cause either acute and lethal or chronic infections in adult zebrafish (van der Sar *et al.*, 2004). Results of micro-array and RNA deep sequencing experiments have provided insights into mycobacterial response genes in the embryo and adult infections (Meijer *et al.*, 2005, Hegedus *et al.*, 2009, van der Sar *et al.*, 2009).

Aims/hypothesis of project

Aims:

- To reproduce, develop and extend the existing zebrafish systems in order to produce tractable models of inflammation in which immune responses can be analysed and modulated.
- To use the zebrafish models developed to investigate the role of p38 MAPK in inflammation and the immune response.
- To investigate the effects of mutations in genes within the Notch signalling pathway on inflammatory responses and development of the haematopoietic compartment in larval and adult zebrafish.

Hypothesis:

Zebrafish can be used as an informative model of mammalian inflammatory processes and reveal novel insights into the role of p38 MAPK and Notch signalling in the immune response.

CHAPTER 2. Materials and methods

Zebrafish care and breeding

Zebrafish Wild Type (WT) fish, transgenic fish were bred according to the Animals Scientific Procedures Act 1986. All fish were maintained according to standard practices (Nusslein-Volhard, 2002). Zebrafish adults were fed on a combination diet of TetraMin flake food and live brine shrimp (ZM Systems). *MPO:GFP* transgenic zebrafish were generated by Dr Stephen A Renshaw (Renshaw *et al.*, 2006). *zpu.1:EGFP* zebrafish were generated by Professor Alistair C Ward (Ward *et al.*, 2003). WT (*AB/Tübingen* (AB/TU) strain), *bea*^{tw212b} and *des*^{tp37} were obtained from Tübingen zebrafish stockcenter.

Zebrafish models of inflammation

In chapter three I have described the development and characterisation of different models of inflammation and their experimental results. There I give further details of experimental procedures used.

Tail transection and tail nick injury, MPO staining of embryos and image acquisition

WT, mutant and transgenic embryos between 4 and 6-days-postfertilization (dpf) were anesthetized by immersion in system water with 4.2% tricaine (Sigma). Tail nick wound injury of the tail was performed by making a small wound using a 19-gauge needle to the ventral median fin posterior to the cloaca. Tail transection wound injury of the tail was performed using a sterile scalpel to transect and remove the distal tip of the tail. Embryos were untreated or exposed to LPS at a concentration of 1 or 10 µg/ml in system water using *Escherichia coli* LPS serotype

O55:B55 or *Salmonella enterica* LPS serotype *typhimurium* (Sigma). The inhibitor SB203580 (inhibitor of p38 MAPK), when used, was used at a concentration of 10 μ M (Sigma). The inhibitor DAPT (inhibitor of γ -secretase), when used, was used at a concentration of 50 μ M (Sigma). The concentrations of inhibitors used were based on previous experiments in the lab which determined effective inhibition versus toxicity.

Embryos for histological staining were incubated at 28.5°C in treatment media for the time indicated then killed with an overdose of tricaine. Embryos were fixed in 4% paraformaldehyde in distilled water for at least 1 h at room temperature before washing with 0.1% Tween 20 in PBS and staining for MPO with 0.5 mg/mL diaminobenzidine, 0.03% hydrogen peroxide in PBS. Embryos were imaged on a Zeiss Axiovert S100 for MPO-stained leukocytes and images captured using Open Lab v3.1 software (Improvision, Coventry, U.K.) and CoolSnap digital camera (Media Cybernetics, Silver Spring, MD).

MPO:GFP embryos used in *in vivo* time course experiments were wounded at time 0, placed in treatment media, then imaged at various time points and scored for GFP positive cells at the wound site.

zpu.1:EGFP transgenic embryos used in time lapse imaging experiments were wounded at time 0, placed in treatment media for 2 hours then transferred to 0.8% low melt agarose (Flowgen, Lichfield, UK) for imaging. Images captured using a Zeiss Axiovert 200 inverted microscope controlled by C-Imaging Simple-PCI acquisition software. The time gap between two consecutive images was 15 seconds.

***Mycobacterium marinum* infection**

WT and DES embryos at 28 hpf were micro-injected in yolk sac with dsRed transformed *Mycobacterium marinum* (strain M, ATCC BAA-535 (Ramakrishnan and Falkow, 1994)). Embryos were micro-injected into the yolk sac using glass capillary micro-injection pipettes and forced air with a Narishige injection station

and micromanipulator with uniform volumes of *M. marinum* culture at an OD concentration of 0.5 and survival was monitored over time.

Human neutrophil preparations

Human neutrophils were prepared from the peripheral blood of healthy volunteers. Blood was separated using dextran sedimentation and a Percoll gradient yielding highly pure human neutrophils (>95%).

WKM (whole kidney marrow) extractions, coelomic cavity lavages and FACS sorting of immune cell populations.

WKM and coelomic cavity cells were isolated from adult zebrafish. WKM and processed as previously described (Traver *et al.*, 2003b, Moss *et al.*, 2009). The cell isolates were washed, and resuspended in ice-cold Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), passed through a 40µm sieve then analysed and sorted by flow cytometry using a FACSVantage with DiVa Option (Becton Dickinson) the myelomonocyte population has been previously described by Traver *et al.* (Traver *et al.*, 2003b).

Extracted cells, when stimulated with LPS, were exposed to varying concentrations of *E.coli* LPS (serotype 055:B5 or 026:B6 as stated) or PMA (10 ng/ml) and visualised/lysed for RNA at the times specified. The inhibitor SB203580, when used, was used at a concentration of 10 µM (Sigma).

Stimulation with various concentrations of LPS (serotype 026:B6) or PMA (at 10 ng/ml) was performed on embryos at 5 days post fertilisation and WKM (cell suspensions prepared as described above) from adult fish and the tissues processed for cDNA.

For phagocytosis assays WKM cell suspensions were incubated at 28.5°C for two hours in IMDM media containing 10% FBS and 0.005 ng/ml 0.5 µM plain green fluorescent beads before collection of cell cultures, centrifugation and re-suspension, followed by cell sorting by flow cytometry.

Development of *in vivo* computational automated cell tracking model; Image processing and data transformation:

The images analysed contained fluorescent PU.1 positive cells. All images were processed in *R* using the package *EBImage*. Cells were detected with an edge detection method using a threshold for the light intensity. Each detected cell was described as an object with the coordinates of its geometrical centre describing the cell location and the occurrence time (Fig. 3a). The cells were tracked over time using a surface-tracking algorithm that is not based on any prior knowledge about the dynamical behaviour. Because the time-lapse microscopy data are optimized in the experimental setup, e.g. 15 sec time gap between two consecutive images, double tracking error was avoided (Beltman *et al.*, 2009). All cell trajectories that included time points in which the cell was located at the edge of the image were excluded. The images contain the zebrafish tail with the whole transection wound and only those trajectories with a distance to the injury of less than 800 μm were included. Only trajectories that contain more than 50 time steps were used for the analysis in order to improve the reliability of the results. Bright field and fluorescent images were produced at each time point to correct for small movements of the zebrafish.

To analyse the extracted image data more efficiently and to combine or compare data from several movies it was necessary to normalize them, e.g. the reorientation of the object positions in respect to the blood vessel of the fish. This was achieved by using linear transformation. The transformation describes the rotation and shifting of the new coordinate bases in the way that the blood flow describes the *y*-axis and orthogonal to it the *x*-axis, which is located approximately parallel to the wound (Fig. 3b). The orientation is based on the bright field images.

Dynamical models

The trajectories obtained were tested for a random walk. The random walk (Hannigan *et al.*, 2001) is described as:

$$\frac{\partial P(x,y,t)}{\partial t} = A \nabla^2 P$$

with the vector A of the diffusion coefficients, and the probability density function $P(x,y,t)$ of the object location at time t . The null hypothesis H_0 states that the tested trajectory shows a random walk. Two aspects of a random walk were analysed. As a first criterion the distribution of the angles between two vectors of the trajectory was tested for uniformity, i.e. whether the motion vectors are isotropic (see **Figure 14** in chapter 4), using the Kolmogorov-Smirnov test. Additionally the distribution of the length of the vectors was investigated. This was done for the entire vector and its projections onto X and Y direction, respectively. These distributions were tested for normality with a Kolmogorov-Smirnov test for normal distributions. The directionality coefficient D was calculated as the coefficient of the shortest distance between the start and end point of a trajectory and the actual length of the trajectory. A D -value close to one indicates highly directed movement and a D -value close to zero indicates that the movement is not directed. Note D -value close to zero does not necessarily imply that the cell performs a random walk as described in equation 1. A permutation test was used for all significance analyses. Juliane Liepe developed this computational automated cell tracking system.

Preparation of RNA and cDNA from Embryos and Adult tissue

Zebrafish embryos were administered a lethal dose of MS-222 (Sigma, see appendix I). MS-222 was removed and embryos were homogenised using a sterile pestle in lysis buffer. Cells were lysed directly in lysis buffer. RNA was isolated from embryos and cells using Magmax™-96 total RNA isolation kit (Ambion Inc) following the manufacturers protocol. The quality and quantity of RNA was assessed spectrophotometrically using a Thermo Scientific Nanodrop™ 1000. For cDNA synthesis, 85 ng of mRNA was reverse transcribed using a high capacity cDNA reverse transcription kit (Applied Biosystems) following the manufacturers protocol. Quantitative RT-PCR was performed with Taqman Fast Universal assays (Applied Biosystems) using a 7500 Fast instrument. The quantitative PCR amplification

program used was as follows, 95°C for 20 s (1 cycle), 95°C for 3 s then 60°C for 30 s (40 cycles). Results were normalized to 18S and calibrated to untreated samples unless otherwise stated. The Taqman gene expression assay IDs for assays used are shown below.

Gene	Taqman Assay ID
<i>csflr</i>	Dr03125167_m1
<i>mpo</i>	Dr03075659_m1
<i>pu.1</i>	Dr03083168_m1
<i>tnfa</i>	Dr03126850_m1
<i>lta</i>	Dr03109563_m1
<i>il1b</i>	Dr03114368_m1
<i>cxcl-C1c</i>	Dr03436643_m1
<i>cxcl12a</i>	Dr03119119_m1
<i>hes1</i>	Custom design

Expression of genes for which Taqman assays were unavailable was determined by SYBR green qPCR analysis and the sequences designed against these transcripts are given below.

Gene	Forward Primer sequence 5'-3'	Reverse Primer sequence 5'-3'
<i>tnfa</i>	CCATTTAACAGGTGGATACAAC	CCATCATCGGGAATGATAATCTCC
<i>il-8</i>	TGTGTTATTGTTTTCTGGCATTTC	CTGTAGATCCACGCTGTTCGC
<i>eflα</i>	GGAAGCCGCTGAGATGGCAAGG	GGTACTTCTCAGGCTGACTGTGC

All primers were checked for hairpins, palindromic sequences and dimers using Net Primer and were used at 0.5 μ M final concentration in PCR reactions. Single product amplification was confirmed by carrying out dissociation (melting) curve analysis post-amplification. Relative gene expression levels are normalised against the housekeeping gene elongation factor 1 alpha (efl- α).

Western blot analysis

Embryos at 4dpf were treated with appropriate media and incubated for one hour at 28.5°C before overdosing with tricaine. Zebrafish tissue samples were harvested from whole embryo tissue using a sterile pestle with detergent free lysis buffer. The lysates were cleared by centrifugation then re-suspended in laemmli buffer containing 5% b-mercaptoethanol and boiled at 100°C for 5 min. Samples were analysed by SDS-polyacrylamide electrophoresis (12%). Samples analysed by SDS-polyacrylamide gels were electroblotted onto nitrocellulose membranes (HybondTM-ECLTM) (Amersham Biosciences). Membranes were stained with ponceau red followed by two brief (5 min) washes with 0.05% Tween-20 in PBS (PBS-T). Non-specific binding was blocked with 5% dried milk (marvel) prepared in 0.05% Tween-20/PBS for one hour at room temperature. The primary antibody used, Anti-ACTIVE® p38 pAb (Promega), was prepared in 5% marvel/PBS-T (1:1000) and incubated with membranes overnight at 4°C. Membranes were washed three times (5 min per wash) with 0.05% Tween-20/PBS (PBS-T). An Anti-rabbit HRP conjugated secondary antibody was prepared in 5% marvel/PBS-T (1:2500) and incubated with membranes for 2 hours at room temperature. Membranes were washed three times then treated with SuperSignalR West Pico chemiluminescent substrate for detection of HRP (Thermoscientific) (prepared following the manufacturers protocol) for 5 min in the dark. All blots were visualised using an ECL detection system (BIO- RAD).

Chapter 3. Characterisation of experimental models of inflammation in zebrafish

Aims and Introduction:

Aims:

- To reproduce and develop the existing zebrafish systems in order to produce tractable models of inflammation in which immune responses can be analysed and modulated.

Introduction:

The p38 MAPKs are a family of stress-activated proteins central to cellular responses activated by external stimuli. They are candidate drug targets for treatment of a broad range of inflammatory diseases including asthma (Adcock *et al.*, 2008), rheumatoid arthritis (Thalhamer *et al.*, 2008) and atherosclerosis (Osman *et al.*, 2008). LPS is a potent activator of p38 MAPK (Sweet and Hume, 1996) that regulates the translation and transcription of the inflammatory cytokines IL-1 and TNF (Adams *et al.*, 2001). Inhibitors of p38 MAPK prevent the translation and transcription of IL-1 and TNF either directly or indirectly via the action of downstream protein MAPKAP K2 (Kumar *et al.*, 2003). The MAP kinases are highly conserved across species and zebrafish possess 2 isoforms of the mammalian p38 α gene. The aim in this chapter is to establish models of acute inducible inflammation in zebrafish to use in later chapters to test the role of p38 MAPK using pharmacological inhibitors.

Several studies have demonstrated that tail fin wounding in zebrafish embryos, including tail transections, medial fin incisions and laser-inflicted wounds induce the migration of leukocytes to the site of tissue damage (Lieschke *et al.*, 2001, Renshaw *et al.*, 2006, Mathias *et al.*, 2006, Brown *et al.*, 2007, Redd *et al.*, 2006). In

particular neutrophil mediated responses to acute wounding have been demonstrated using a combination of histochemical staining of fixed embryo tissue for the neutrophil specific marker MPO and transgenic zebrafish lines expressing fluorescent proteins under the MPO promoter (Renshaw *et al.*, 2006, Mathias *et al.*, 2006, Loynes *et al.*, 2009). Here the aim was to add to the complexity of these models of injury induced acute inflammation by introducing other inflammatory signals, such as exposure to LPS, and determine their effect on the resulting leukocyte recruitment. The acute wound injury models developed in this chapter will be used in chapter four to investigate the differential effects of inhibiting p38 MAPK.

The search for lineage specific markers in the innate immune system in zebrafish embryos has been a goal of many research groups in recent years. The complex overlap in expression of various candidate markers has led to controversy over the definition of macrophage and neutrophil cell types specifically. The lack of tools, e.g. monoclonal antibodies, for identifying leukocytes in zebrafish has compounded this problem. However the use of histochemical staining and the production of several transgenic lines of fish expressing fluorescent proteins under the control of potential lineage specific markers has allowed distinctions between innate immune cells to be made. Transgenic lines with promoter driven fluorescent cell types include the *pu.1*, MPO, *lyzC*, and *Fli1* (Ward *et al.*, 2003, Mathias *et al.*, 2006, Renshaw *et al.*, 2006, Hall *et al.*, 2007, Lawson and Weinstein, 2002) and here I describe the studies that have revealed how expression of these and other cell markers define specific leukocyte cell types in zebrafish. Herbomel *et al.* first described *l-plastin* as a haematopoietic marker gene for macrophages (Herbomel *et al.*, 1999). Bennett *et al.* used *in situ* hybridisation techniques to determine the expression and co expression of the haematopoietic markers marker *mpo*, *l-plastin* and *pu.1* (Bennett *et al.*, 2001). *l-plastin* expression was localised to anterior yolk region of early embryos which was consistent with the findings of Herbomel *et al.* (Herbomel *et al.*, 1999, Bennett *et al.*, 2001). Double *in situ* hybridisation determined the presence of cells that coexpressed *mpo* and *pu.1* but no cells that coexpressed *mpo* and *l-plastin*. This distinct expression of *mpo* and *l-plastin* indicated *mpo* expression was specific to granulocytes and that *l-plastin* expression was specific to macrophages (Bennett *et al.*, 2001). Coexpression of *mpo* and *pu.1* was transient

indicating a role for *pu.1* expression in regulation of the development of myeloid progenitors into mature neutrophils (Bennett *et al.*, 2001). Lieschke *et al.* used MPO histochemical staining to stain granulocytes in zebrafish embryos, which they termed heterophils, and which were functionally similar to mammalian neutrophils (Lieschke *et al.*, 2001). *mpo* expression and morphological characteristics in cells of the zebrafish adult kidney and embryonic myelopoeitic regions confirmed in zebrafish *mpo* expression is restricted to cells of the granulocyte lineage as it is in humans (Bennett *et al.*, 2001). The MPO:GFP transgenic zebrafish line was produced and described by two groups independently in 2006 (Mathias *et al.*, 2006, Renshaw *et al.*, 2006). Both groups produced fish expressing GFP under the control of the MPO promoter and based on previous descriptions of MPO as a neutrophil-restricted granule protein they both defined these MPO expressing cells as neutrophils.

Lui *et al.* discovered the lysozyme C gene (*lyzC*) another marker for the myeloid lineage cell types in the embryo (Liu and Wen, 2002). They defined this *lyzC* gene as a macrophage specific marker on the strength of it being co-expressed with *pu.1* and *l-plastin* genes. However as both of these genes are broad myeloid lineage markers this is now considered an over interpretation of the results and a macrophage specific marker remained to be found. *lysozyme C* as a candidate macrophage lineage marker was further investigated in 2007 when Hall *et al.* produced a transgenic zebrafish line expressing EGFP and DsRED2 under the *lysozyme C* promoter (Hall *et al.*, 2007). Hall *et al.* scrutinised the expression profiles of the fluorescent *lysozyme C* expressing cells in these transgenic fish and found labelled cells expressed markers of the macrophage lineage including *l-plastin*. However they also detected *mpo* transcripts within some of these cells and so concluded the *lysC::EGFP* and *lysC::DsRED2* reporter lines as having marked myelomonocytic compartments (Hall *et al.*, 2007). Meijer *et al.* used a transgenic zebrafish line showing leukocyte specific YFP expression, generated from a large-scale enhancer detection screen in the zebrafish genome (Ellingsen *et al.*, 2005), to re-examine the specificity of described myeloid markers. This myc/YFP-expressing cell type in the zebrafish embryo was determined to mark a subset of granulocytes and by carrying out co-localization analysis they reexamined existing markers and

that showed *l-plastin* can be considered a general leukocyte marker, *csflr* can be considered a macrophage specific marker, and *mpx* and *lysozyme C* can be considered neutrophil specific markers (Meijer *et al.*, 2008). Meijer *et al.* point out that caution should be employed when using the described myeloid marker genes in zebrafish. Temporal considerations must be made as co localization of different cell markers can change over time, for example cellular co-localization of *l-plastin* and *mpx* was found to reduce over time which could explain why other groups did not observe overlap in expression of these genes (Meijer *et al.*, 2008). Currently whilst *mpx* can be described as neutrophil specific and *csflr* can be described as macrophage specific all other markers share some form overlap with these markers and so are less specific markers of the myeloid compartment and must be used in combination with other markers when describing cell type. Here I report cell recruitment to the acute injury models in zebrafish embryos using a combination of histochemical staining and transgenic fish expressing fluorescent proteins under lineage defining promoters.

The unique combination of optical translucency, which allows *in vivo* imaging of host responses in real-time, and genetic tractability, allowing the production of transgenic fish expressing fluorescent proteins under lineage specific promoters, have made zebrafish invaluable in the study of host/pathogen interactions to a variety of infectious organisms including mycobacteria (Davis *et al.*, 2002a, Prouty *et al.*, 2003, Clay *et al.*, 2007, van der Sar *et al.*, 2006, Stockhammer *et al.*, 2009). *M. marinum* is a strain of mycobacterium closely related to *Mycobacterium tuberculosis* and causes tuberculosis in ectotherms using similar pathogenic mechanisms (Clark and Shepard, 1963, Clay *et al.*, 2007). Here I report a mycobacterial infection model in zebrafish embryos that is comparable to those reported by others (Davis *et al.*, 2002a, Clay *et al.*, 2007). I have used this infection model to study the role p38 MAPK and defects in members of the Notch signalling pathway on survival and present the results of these experiments in subsequent chapters.

The immune system of adult zebrafish has been characterised in several studies. The WKM is the haematopoietic organ in all adult teleosts including zebrafish (Zapata, 1979) and was characterised in zebrafish by Traver *et al.* who isolated each of the major blood lineages by flow cytometry (Traver *et al.*, 2003b).

Adult immune cells including granulocytes, lymphocytes and non-specific cytotoxic cells (NCCs), can also be isolated from adult zebrafish by lavaging the coelomic cavity (Moss *et al.*, 2009). In this chapter I describe the isolation and characterisation of cell populations from the WKM and coelomic cavity of adult fish, which I have used in subsequent chapters to analyse zebrafish immune cell responses to inflammatory stimuli and pharmacological inhibitors.

Here I will introduce and characterise the various models that will be used throughout this thesis to examine the role of p38 MAPK and Notch signalling in the zebrafish immune response.

Results

Acute wound injury models: tail nick and tail transection

Kinetics and quantitative analysis of leukocyte recruitment/retention

Using fixed and MPO stained (neutrophil selective marker) WT embryos at 5 dpf I demonstrate that a tail nick injury in the ventral fin or tail transection injury of the distal tip of the tail induced neutrophil recruitment to the site of the wound injury (Figure 1 A & B respectively). Neutrophil recruitment to a tail nick wound injury is highest (5 cells) at 2 hours post injury (hpi) and resolution of neutrophilic inflammation is largely complete (0 cells) by 6 hpi (Figure 3 A). Recruitment to a tail transection injury is highest (6 cells) at 4 hpi and by 8 hpi the number of MPO positive cells at the site of injury is reduced (3.5 cells) (Figure 3 B).

Effects of wound size/depth and LPS on leukocyte migration

Both the size and depth of tail nick wound injuries influence cell migration and/or retention. Embryos were nicked midway along the ventral median fin and the wounds were scored small, medium and large according to the depth of the cut. Small wounds (Figure 2 A) are defined as incisions that go to a depth no greater than 25% the distance between the outside edge of the fin and the vasculature. Medium wounds (Figure 2 B) are defined as incisions that go to a depth of greater than 25% and no greater than 75% the distance between the outside edge of the fin and the vasculature. Large wounds (Figure 2 C) are defined as incisions that go to a depth greater than 75% the distance between the outside edge of the fin and the vasculature. The level of neutrophil recruitment is associated with the size of the tail nick wound inflicted. There is a hierarchical neutrophil recruitment with large

wounds > medium wounds ($P < 0.01$) > small wounds (Figure 2 D). For this reason only wounds classed as medium sized were included in any data analysis to avoid any bias caused by variations in wound size.

Having determined the peak time for neutrophil recruitment, in unstimulated fish with wound injury I investigated the effect of the bacterial derived cell wall component LPS. In both models of injury, tail nick and transection, stimulation with LPS resulted in an increase in cell recruitment at the peak time points 2 and 4 hpi respectively (Figure 4 & Figure 5). I demonstrate that in the presence of LPS there is an increase in the number of neutrophils migrating to an acute tail nick (Figure 4 A & B). Immersion of embryos wounded by tail nick injury in *E.coli* LPS significantly ($P < 0.001$) enhanced neutrophil 2 fold (Figure 4 A & B). Similarly the immersion of embryos wounded by tail transection in *E.coli* LPS significantly ($P < 0.001$) enhanced neutrophil recruitment/retention (Figure 5 A & B).

Injury in transgenic embryos allows monitoring of leukocyte recruitment in individual live embryos over time.

Using transgenic zebrafish expressing GFP under the neutrophil specific MPO promoter (Renshaw *et al.*, 2006) I demonstrate that LPS at 1 $\mu\text{g/ml}$ significantly enhanced recruitment of neutrophils post injury in live embryos (4 & 5 dpf) at 4, 5 and 6 hrs ($P < 0.05$) when compared to cell migration in response to wounding alone. By 24 hrs the number of neutrophils at the site of injury is reduced (Figure 6 A). During the first 4 hrs of injury the recruitment/retention of cells was significantly greater in the fish exposed to LPS (the slopes of the linear regression lines were significantly different, $p < 0.02$; (Figure 6 B). In the presence of LPS I noted that there was an increase in the number of neutrophils recruited to and/or retained at the site of injury but that the kinetics of the migration remained the same as that observed in wounding alone, i.e. there was no change in the peak time point at which maximal recruitment was observed (Figure 6 A).

I confirmed that it was also possible to follow recruitment of leukocytes to a tail transection injury in the myeloid lineage specific *zpu.1:EGFP* transgenic embryos, as was possible with the *MPO:GFP* fish. Time lapse microscopy images of recruitment to a tail transection wound demonstrates fluorescent immune cells are visible and can be seen migrating in the injured tail (Figure 7). The model of cell recruitment and tracking of cells in *zpu.1:EGFP* transgenic embryos is further developed in chapter four of this thesis where the effect of LPS exposure is comprehensively investigated.

Embryo infection models

Development of *M. marinum* infection model in zebrafish embryos

In order to develop and characterise a model of embryonic bacterial infection in zebrafish dsRED *M. marinum* was micro-injected into the yolk sac of embryos at 24 hpf (Figure 8 A). The spread of the bacterial infection throughout the embryo was visualised (Figure 8 B, D & C) and survival time monitored (Figure 8 E). At 24 hpi dsRED *M. marinum* was visible within the yolk sac and by 72 hpi was disseminated throughout the embryo and present in the tissues. By 72 hours bacteria are concentrated in aggregates within the vascular bed of the tissue of the tail posterior to the yolk sac (Figure 8 D). Embryos injected with *M. marinum* survive until 4 dpi when they begin to die and by 6 dpi there are none surviving. In contrast, the survival rate of control embryos injected with PBS is higher with 75% of injected fish alive at 6 dpi (Figure 8 E). As a result of trauma caused by micro-injection ~20% of PBS injected embryos die within the first 2 dpi (Figure 8 E).

M. marinum is disseminated throughout zebrafish embryos by migrating macrophages when injected via the caudal vein (Clay *et al.*, 2007). Transgenic zebrafish expressing eGFP under the myeloid lineage specific Pu.1 promoter were injected in the yolk sac with dsRED *M. marinum*. At 24 hpi several dual positive (Pu.1⁺ and dsRED *M. marinum* positive) were visible outside but close to the yolk

sac (Figure 9 A). However, by 48 hpi many dsRED positive cells were visible throughout the tail and of these a number were also positive for GFP indicating they had been engulfed by Pu.1 positive leukocytes (Figure 9 B). dsRED positive cells were also disseminated throughout the head at 48 hpi but due to the high GFP expression in the yolk sac and the greater depth of the tissue it is difficult to determine the dual positive cells in this region of the embryo (Figure 9 C). At 6 dpf 95% of *zPu.1:eGFP* transgenic embryos injected with *M. marinum* are dead compared with 15% of control embryos injected with PBS only (Figure 9 D) which is consistent with the rate of survival in the WT fish.

Isolation and characterisation of immune cell populations from adult zebrafish

Isolation and characterisation of cells from adult WKM

The haematopoietic organ in the adult zebrafish is the WKM. This organ can be dissected from adult zebrafish and processed to produce cell suspensions (Traver *et al.*, 2003b). Here cell suspensions were produced from WT adult zebrafish, analysed by flow cytometry, and sorted according to forward and side scatter with the aim of isolating a monocyte/macrophage population. Five populations were sorted (Figure 10 A) and cytopspins performed in order to characterise the morphology of the cells in each population. Two large populations within the red gate contain mature erythrocytes. Cells within the green gate displayed granulocyte/monocyte characteristics. The blue gate contains only lymphocytes and the purple gate contains mainly precursor cell types and with some lymphocyte and granulocyte/monocyte cell types (Figure 10 A). The populations identified and characterised in this study correspond with the findings of Traver *et al.* who established that there are two populations of cells of low forward scatter identified as erythrocytes, a population of intermediate forward scatter (FSC) and low side scatter (SSC) identified as lymphocytes, a population with intermediate FSC and

intermediate SSC identified as precursors and finally a population of high FSC and high SSC identified as myelomonocytes (Traver *et al.*, 2003b).

It is possible to sort a mixed population of myeloid cells from zebrafish whole kidney using forward and side scatter. In order to isolate a purer population of macrophage-like cells, with a view to carrying out *in vitro* *M. marinum* phagocytosis and expression assays, the WKM cell suspensions were sorted based on their ability to phagocytose fluorescent beads. WKM cells were isolated and the cell suspensions were cultured with 500 nM fluorescent Latex beads at 28.5°C for 2 hours before sorting for fluorescent positive cells. The fluorescent positive cell population was analysed microscopically and whilst a significant population of the sorted cells had taken up beads by phagocytosis, largely mononuclear phagocytes (Figure 11 A), there was a contamination from other cell types negative for fluorescent beads. In order to optimise the enrichment for phagocytic cells those cells that exhibited high fluorescence, containing two or more beads, were gated and sorted. Those cells that had low fluorescence or were negative for fluorescence were also sorted and the three populations were then analysed by flow cytometry a second time to determine the purity of these cell populations. This analysis revealed that the population originally sorted as negative for fluorescence remained 99.9% negative. The population sorted for low fluorescence had lost much of the positive fluorescence; only 6% of the cells had retained positive fluorescence. Of the highly positive population 25% of the cells sorted as positive retained their fluorescence in the test sort (Figure 11 B). Microscopic examination confirmed that 25% of the cells sorted as highly positive had engulfed the beads. Total sorted cell number in this GFP high population was low (< 200 000) and whilst there were enough cells to perform cytospin analysis the yield for total RNA isolated was very poor (< 0.1 µg) (Figure 11 C). Total sorted cell number in populations that were sorted on forward and side scatter alone, myelomonocytes, lymphocytes, precursors and erythrocytes, were higher (>600 000, >1500 000, >900 000 & >500 000 respectively (Figure 11 C). The myelomonocytic population of interest produced a higher, but still low, amount of total RNA (< 1.0 µg) (Figure 11 C).

Phenotypic characterisation of cells isolated from coelomic lavage of adult zebrafish

Leukocytes are present in the coelomic cavity of adult zebrafish (Moss *et al.*, 2009). The coelomic cavities of adult WT fish were flushed to produce cell suspensions that were subsequently analysed by flow cytometry. The forward and side scatter profiles produced were similar to those described by Moss *et al.* (2009). A population of cells with FSC^{high} and SSC^{high} (Figure 12 A red gate) was identified which, when stained with Wright Geimsa stain, was enriched for myelomonocytes and macrophage-like cells. Two populations of SSC^{low} cells were found and these were composed of small cells with high nuclear:cytoplasm ratios. The positions of these two populations in the FSC confirmed that the SSC^{low}/FSC^{low} subset is the NCC containing population and that these cells with the SSC^{low}/FSC^{intermediate} phenotype are the lymphocyte containing population when classified according to the findings of Moss *et al.* (2009) (Figure 12 A blue and green gates). However, in contrast to the results of Moss *et al.* (2009) I did not identify an erythrocyte population at the FSC^{high}/SSC^{intermediate} position.

Relative expression of lineage markers in sorted WKM and coelomic cavity cells

In order to further characterise the cell types sorted from adult WKM and coelomic cavity, expression of lineage specific markers was carried out. Relative expression of macrophage specific cell marker *csflr* was assessed in WKM sorted cell types and found to be most highly expressed in the precursor group with >25 fold increase in expression than both the myelomonocyte and lymphocytes populations (Figure 13). Relative expression of neutrophil specific cell marker *mpo* was found to be most highly expressed in the WKM derived myelomonocyte cell type with >450 fold increase in expression relative to the lymphocyte with expression in the precursor population 35 fold higher than the lymphocyte population (Figure 13). Expression of pan myeloid marker *pu.1* was found to be most highly

expressed in the WKM derived myelomonocytic population, >6 fold above the level of expression in the lymphocyte cell type, and expression in the precursor cell type was 2 fold above that of the lymphocyte cell type (Figure 13). Relative expression of macrophage specific cell marker *csf1r* was assessed in coelomic cavity sorted cell types and found to be equally highly expressed in the macrophage/granulocyte and the NCC cell types with >6 fold increase in expression above the lymphocyte population (Figure 13). Relative expression of neutrophil specific cell marker *mpo* was found to be most highly expressed in the coelomic cavity derived NCC cell type with >200 fold higher expression relative to the macrophage/granulocyte and lymphocyte cell types (Figure 13). Expression of pan myeloid marker *pu.1* in the coelomic cavity derived cell types was found to be equally highly expressed in the macrophage/granulocyte and the NCC cell types with >6 fold increase in expression above the lymphocyte population expression in the precursor cell type was 2 fold above that of the lymphocyte cell type (Figure 13).

Figures

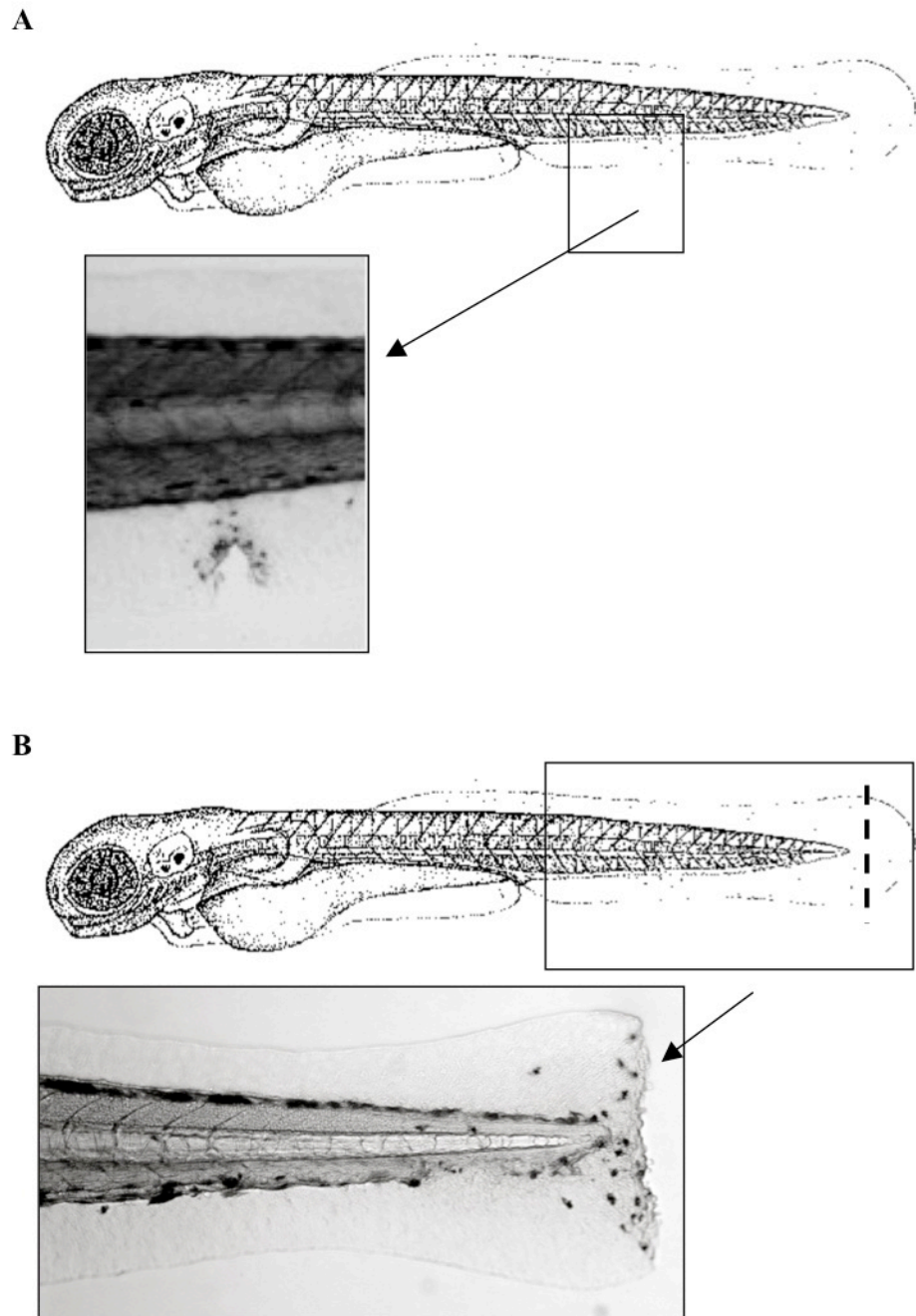


Figure 1. Diagram and image of MPO stained tail nick (A) and tail transection wound (B). Embryos wounded at 5 days post fertilisation (dpf) and incubated for 2 (A) and 4 (B) hpw before fixing in 4% paraformaldehyde and staining for MPO.

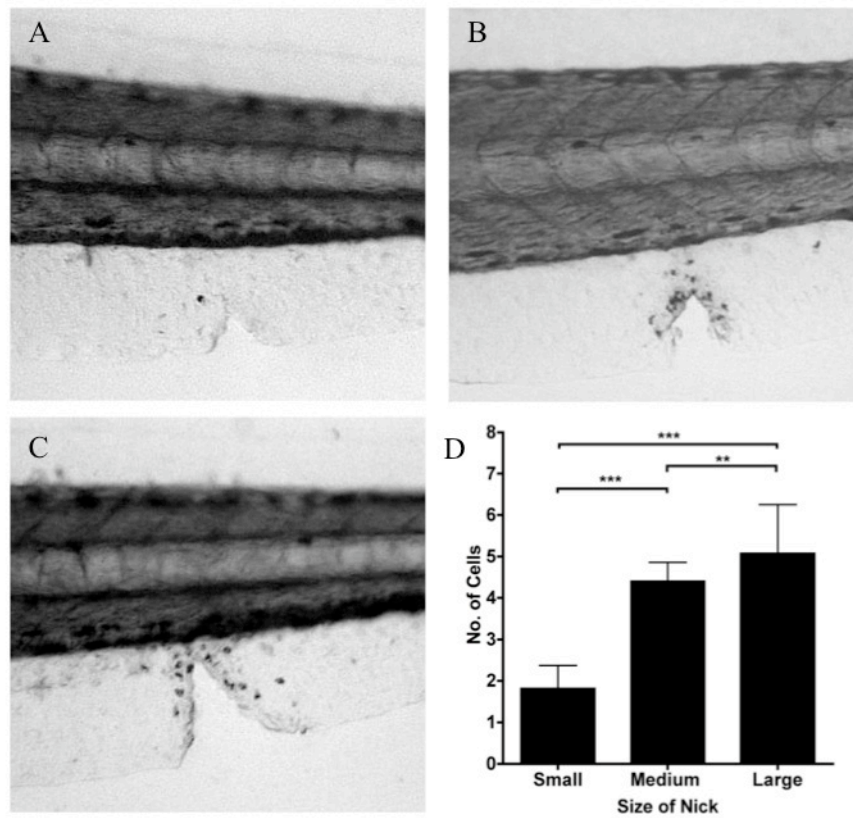


Figure 2. Examples of representative tail nick wound sizes in embryos (2 hpi) fixed and stained for MPO activity, small (A), medium (B) and large (C) and graph showing median number of MPO stained cells recruited against wound size in the presence of 1 µg/ml LPS (D) (n = >90 embryos per bar, error bars are 95% CIs).

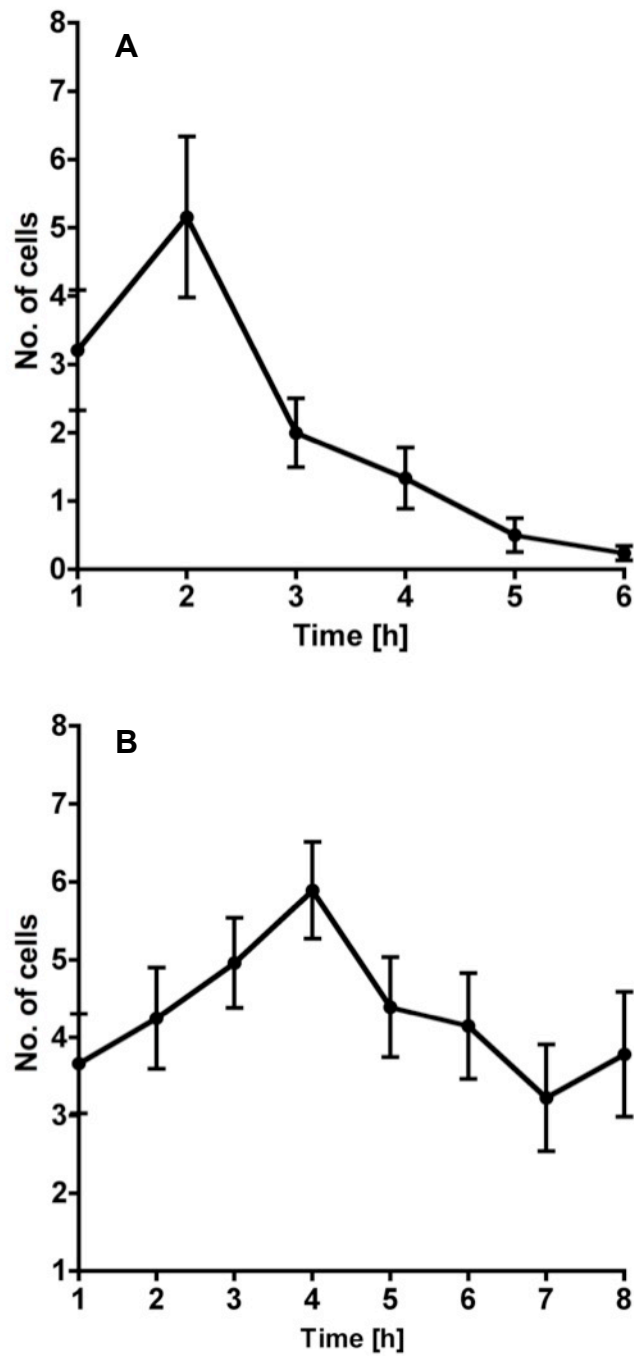


Figure 3. Time course showing recruitment of MPO stained cells to tail nick (A) and tail transection injury (B). Embryos at 5dpf were wounded, incubated in system water then killed and fixed at various time points post wounding and histochemically stained for the neutrophil marker MPO ($n > 15$ embryos per data point, error bars are SEMs).

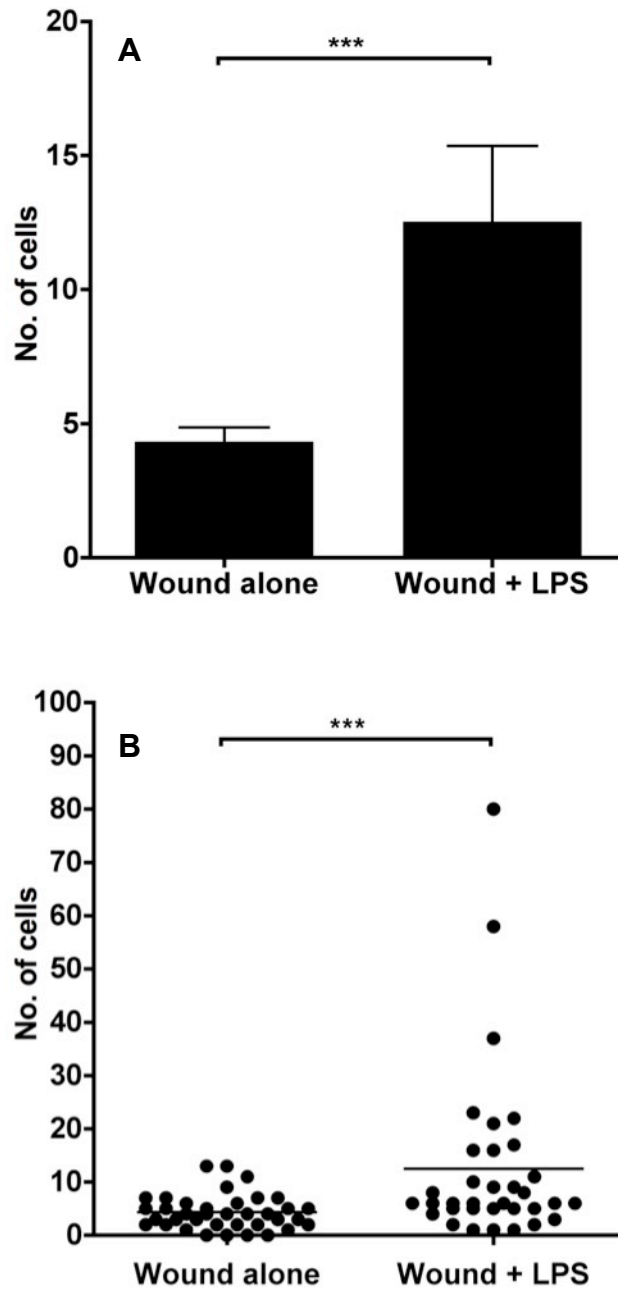


Figure 5. LPS treatment enhances recruitment of MPO stained cells to a tail transection wound. Embryos at 5 dpf were wounded and immersed in system water alone or system water containing *E.coli* LPS (serotype 055:B5) at 1 µg/ml. Graphs represent one data set presented as a bar chart (A) and a dot plot (B) (n = >30 embryos per bar, error bars are 95% CIs).

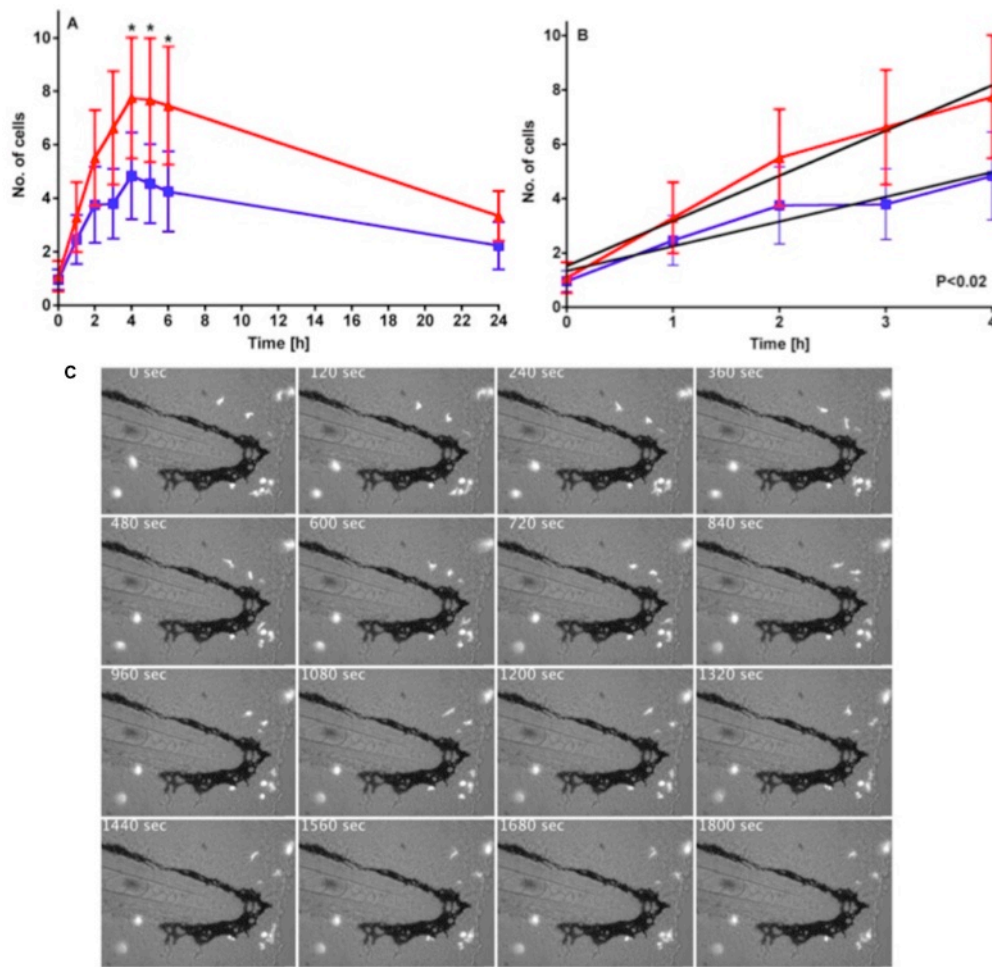


Figure 6. LPS treatment enhances recruitment of neutrophils to a tail wound in *MPO:GFP* zebrafish embryos (3 & 4 dpf). Embryos were wounded then immersed in system water (■) or *E. coli* (eLPS, 055:B5) (▲) at 1 $\mu\text{g}/\text{ml}$ and MPO:GFP positive cells recruited to the wound were recorded over time. Time course over 24 hours showing recruitment of fluorescent cells is recorded over time and analysed using Mann Whitney test (A). The same time course data up to 4 hours presented with linear regression lines. The slopes of the linear regression lines were significantly different, $p < 0.02$ (B). ($n = >20$ embryos per data point, error bars are 95% CIs). Bright field and GFP time-lapse image montage of a tail transection injury in transgenic *MPO:GFP* embryo starting at 1 hpw with 120 sec gaps between images (C).

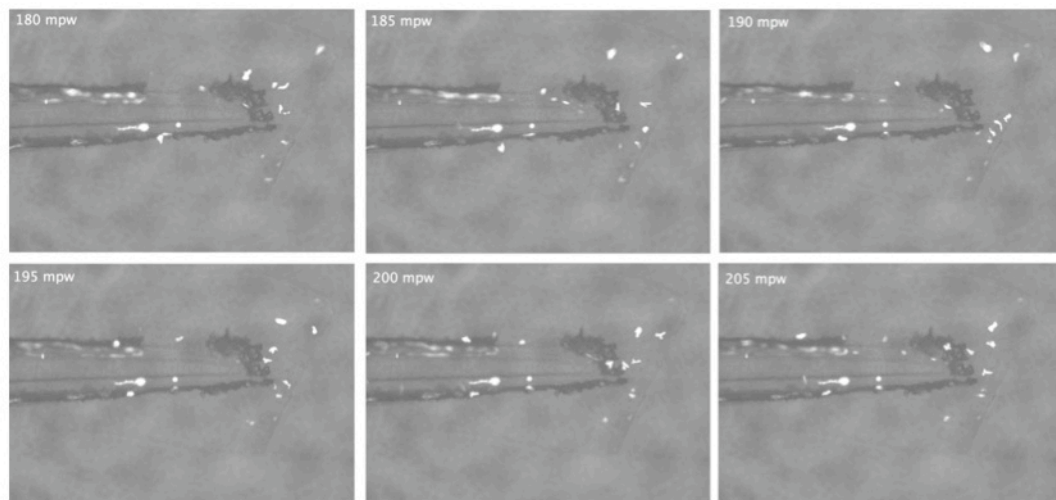


Figure 7. Bright field and GFP time-lapse image montage of a tail transection injury in transgenic *zpu.1:EGFP* embryo starting at 3 hpw with 5 min gaps between images.

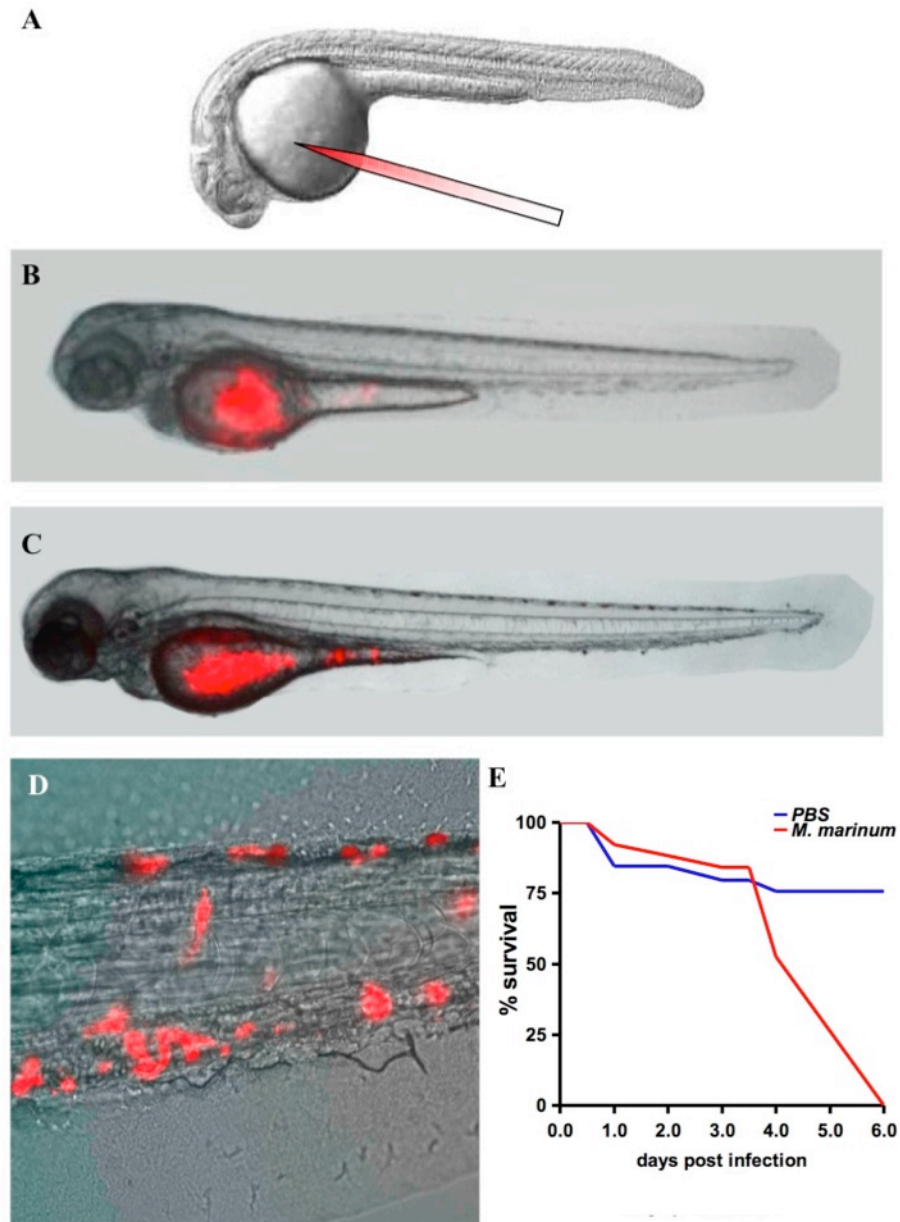


Figure 8. WT Embryos infected with dsRED *M. marinum*. Schematic illustrating location of injection of *M. mariunum* in yolk sac at 24 hpf (A). Image of embryo infected with dsRED *M. marinum* localised to yolk sac at 24 hpi (B). Image of embryo infected with dsRED *M. marinum* localised to yolk sac at 48 hpi (C). Image of embryo infected with dsRED *M. marinum* localised in aggregates within the vascular bed of the tissue of the tail posterior to the yolk sac at 72 hpi (D). Percent survival of WT embryos micro-injected with either PBS of dsRED *M. marinum* at 24 hpf with >35 embryos per condition (E).

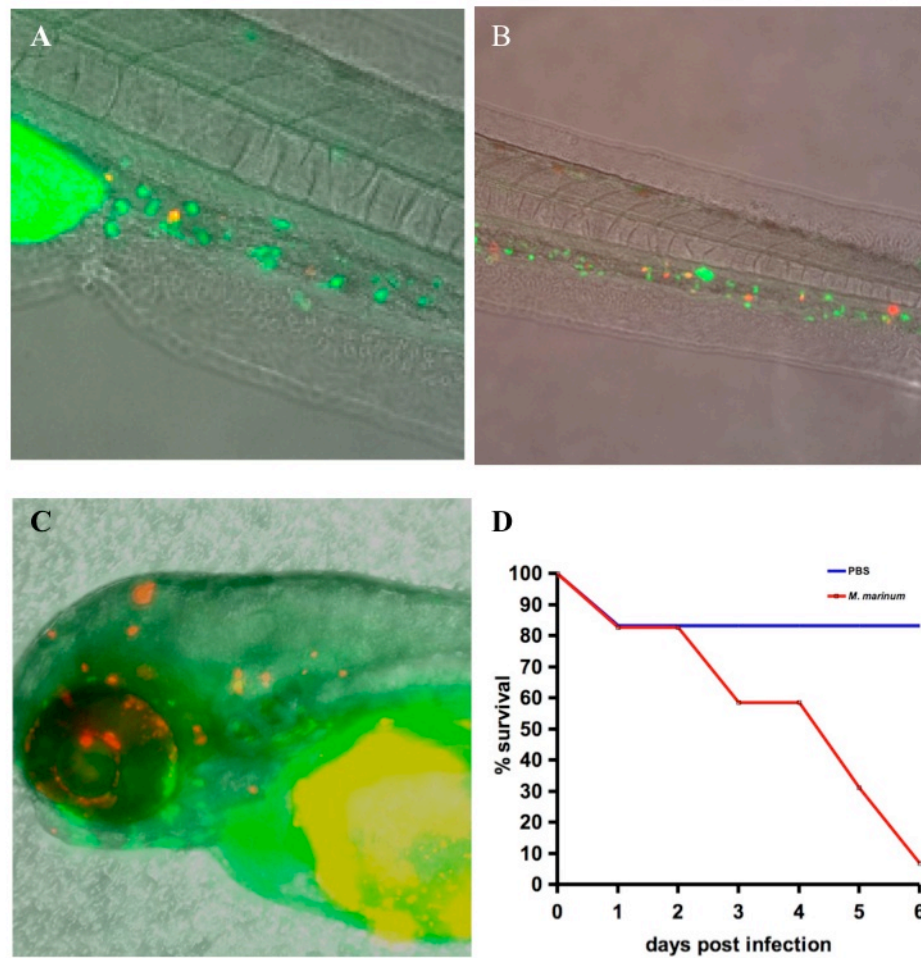


Figure 9. Infection of *zpu.1:EGFP* transgenic embryos with dsRED *Mycobacterium marinum*. Image of cloaca region of the tail of an infected *zpu.1:EGFP* embryo at 24 hpi showing GFP Pu.1 positive cells and two dual positive (eGFP and deRED) cells (A). Image of the tail of an infected *zpu.1:EGFP* embryo at 4 hpi showing eGFP Pu.1 positive cells, dsRED positive cells and dual positive cells (B). Image of head of an infected *zpu.1:EGFP* embryo at 48 hpi showing disseminated dsRED positive cells (C). Images chosen are representative of embryos at each stage of infection. Percent survival of *zpu.1:EGFP* embryos micro-injected with either PBS or dsRED *M. marinum* at 24 hpf with >12 embryos per condition (D).

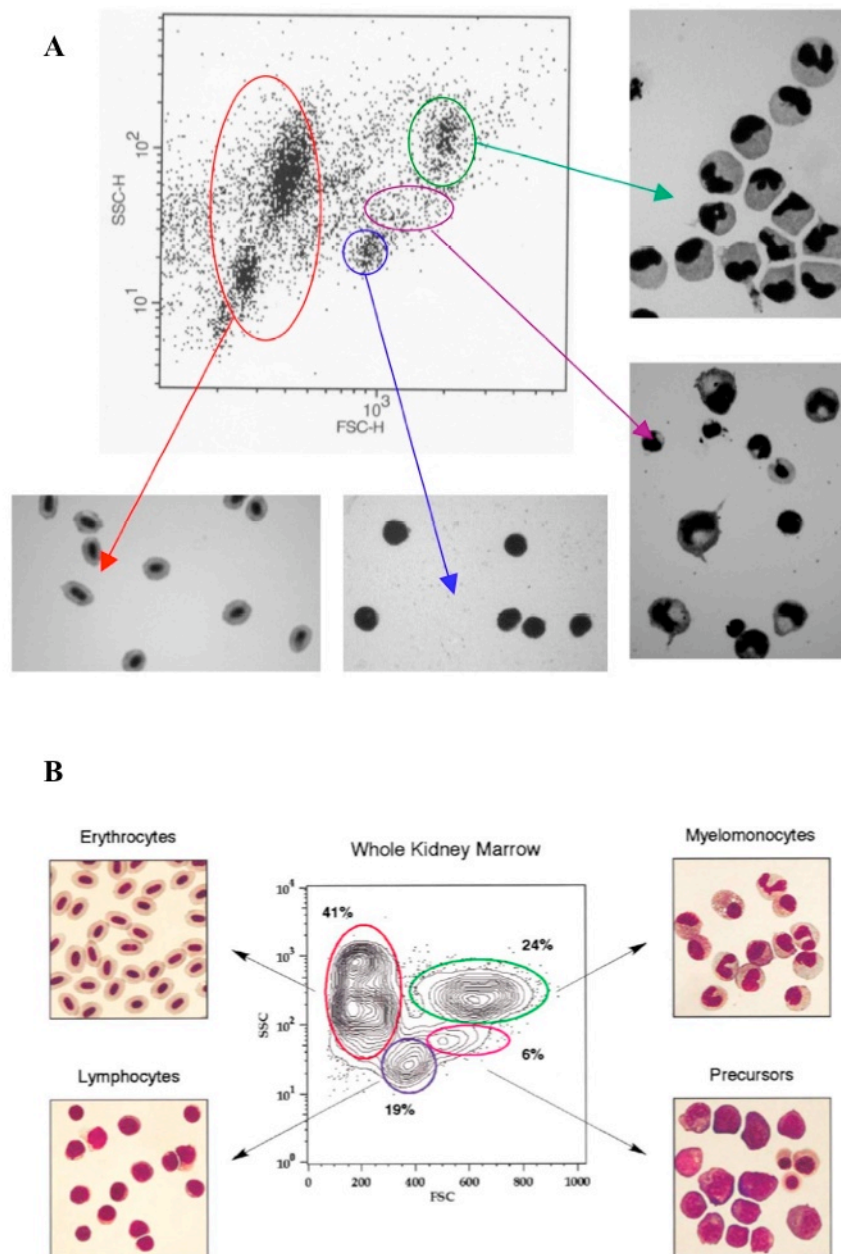


Figure 10. FACS profile of WKM cell suspensions and cytopins showing sorted cell population morphology. Cell types sorted are erythrocytes (red gate) lymphocytes (blue gate) precursor cell types (purple gate) and myelomonocytic cells (green gate) (A). FACS profile of WKM cell suspensions and cytopins showing sorted cell population morphology taken from Traver et al. [2003b] (B).

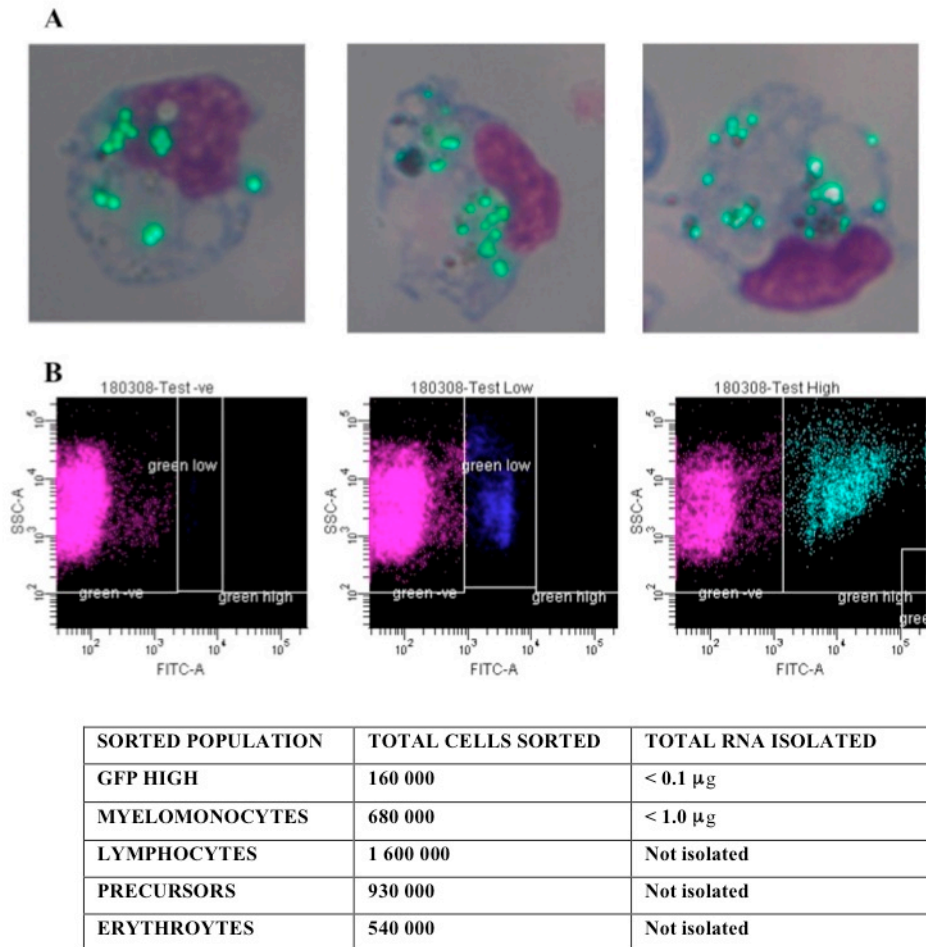


Figure 11. Enriched populations of mononuclear phagocytes can be isolated by flow cytometry. WKM cell suspensions were incubated with 0.5 micro fluorescent beads for 2 hours and then sorted by flow cytometry into 3 populations (negative, low and high) according to their fluorescence. The sorted populations were then passed through the flow cytometer again to determine the level of fluorescence retained by the cells post sorting. FACS plots indicating the proportion of cells that retained their fluorescence post sorting in the negative, low and high sorted populations (A). Cytopspins of sorted WKM cells that have phagocytosed the fluorescent beads (B). Table indicating total cell counts and amounts of RNA purified from populations sorted by flow cytometry (C).

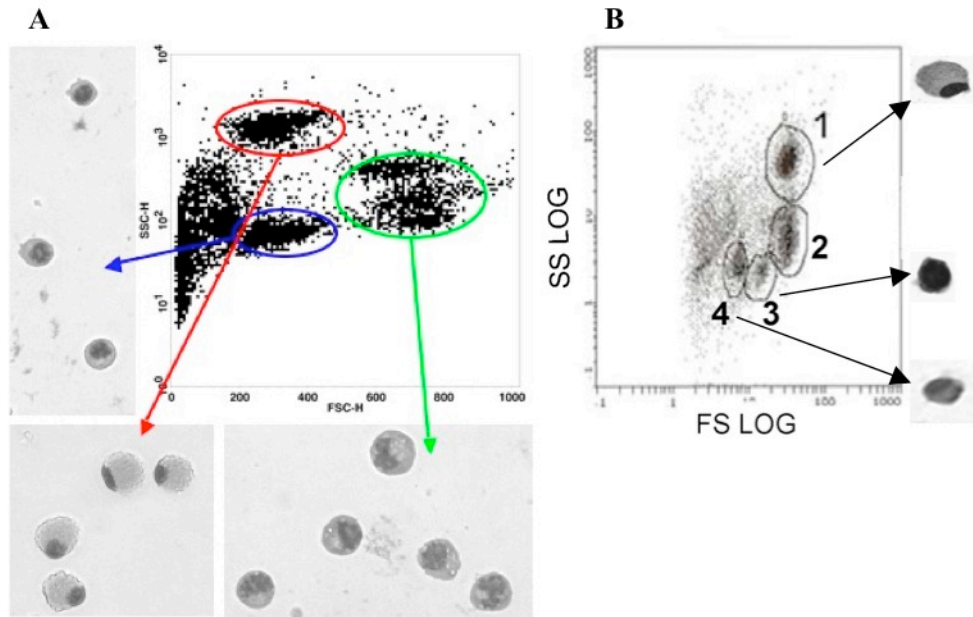


Figure 12. Flow cytometry plot showing populations of cells isolated from the coelomic cavity of adult zebrafish. Cell types sorted are myelomonocyte/granulocyte containing gate (red) nonspecific cytotoxic cell (NCC) containing gate (blue) lymphocyte containing gate (green). Flow cytometry profile of coelomic cavity cell suspensions showing and cytopins showing sorted cell population morphology. Population 1 (FSC^{high}/SSC^{high}) is described as granulocyte containing population. Population 2 ($FSC^{high}/SSC^{intermediate}$) is described as the erythrocyte containing population. Population 3 ($FSC^{intermediate}/SSC^{low}$) is described as the lymphocyte containing population. Population 4 (FSC^{low}/SSC^{low}) is described as the NCC containing population. Adapted from Moss *et al.* (Moss *et al.*, 2009) (B).

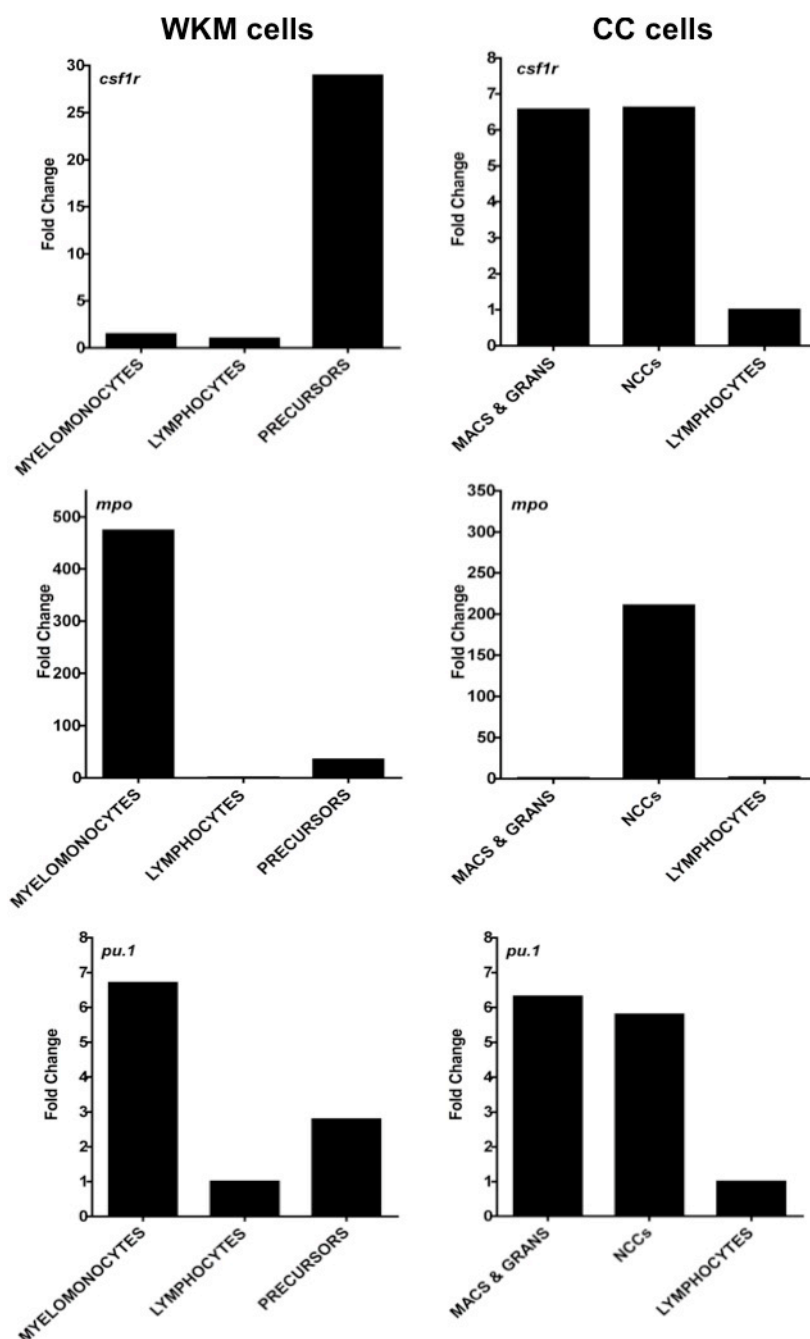


Figure 13. Quantitative PCR analysis of expression of the zebrafish lineage specific cell markers *csf1r*, *mpo* and *pu.1* expression in sorted cell populations from adult WKM tissue and coelomic cavity cells. WKM and coelomic cavity cells extracted from adult fish were sorted as described and cells lysed and processed for cDNA. Gene expression is normalised to 18S and is presented as relative to the population expressing lowest levels of transcript.

Discussion

In this chapter I have established and characterised the various models of inflammation that will be used throughout this thesis to examine the role of p38 MAPK and Notch signalling in the zebrafish immune response.

Acute wound injury models: tail nick and tail transection

I have investigated the neutrophil recruitment response in two previously described types of zebrafish embryonic tail fin injury, the tail nick injury (Mathias *et al.*, 2006, Brown *et al.*, 2007, Cvejic *et al.*, 2008) and the tail transection injury (Renshaw *et al.*, 2006) with the aim of extending these models to include additional inflammatory stimuli, and then to apply these models to study the dynamics of leukocyte recruitment and its dependency on p38 MAPK. The number of neutrophils recruited to the injuries was comparable to that of work published by previous groups. The peak of neutrophil recruitment in the tail nick model was 2 hpw with a mean total of 5 cells which is comparable to results presented by Cvejic *et al.* who recorded an average of 7 neutrophils at the wound site at 1.5 hpw (Cvejic *et al.*, 2008). The peak time of neutrophils migration in tail transection wounds was 4 hpw and confirms the findings of Renshaw and colleagues. However the magnitude of the response they observed was greater, ie. a mean cell number of 10 cells at 4 hpw (Renshaw *et al.*, 2006). It is possible that this difference reflected variation in severity of wounding. Renshaw *et al.* performed a complete transection of the tail severing the blood vessels in the vascular tissue at the distal tip of the tail. Here I transect only the distal tip of the fin avoiding damage to the vasculature. This less severe injury results in recruitment of fewer neutrophils but allows greater accuracy in the counting of the recruited neutrophils, which can be difficult to count individually when very high numbers are observed. When higher numbers of cells are recruited in more severe injury (Renshaw *et al.*, 2006) the cells overlap and it is

necessary to approximate cell number by quantifying the overall size of the fluorescent area.

I demonstrate *in vivo* that LPS enhances the number of recruited neutrophils to a site of injury. LPS induced elevated numbers of MPO cell counts in tail nick and transection injuries as measured by staining of fixed embryos and imaging of injuries in live transgenic *MPO:GFP* embryos over time. The presence of bacteria in the water of wounded embryos has been shown to enhance leukocyte recruitment to wound injury in zebrafish embryos (Brown *et al.*, 2007). Here I show that the bacterial component LPS alone is sufficient to induce a significant enhancement in neutrophil recruitment to wound injury. Neutrophil recruitment has been investigated in other models of LPS mediated inflammation such as LPS induced lung injury (Chignard and Balloy, 2000). However in this *in vivo* study I report that LPS can induce an increase in neutrophilic inflammation at sites of surgical wound injuries. It is likely that this LPS mediated increase in neutrophil numbers is due to an increase in neutrophil viability since Loynes *et al.* reported a reduction in apoptosis mediated by LPS in a similar wounding model (Loynes *et al.*, 2009). LPS induces inflammatory responses in fish models of inflammation (Iliev *et al.*, 2005a, Watzke *et al.*, 2007, Sepulcre *et al.*, 2009). However whilst LPS recognition in mammals by TLR4 is well characterised LPS recognition in fish is poorly understood. It has recently been determined that the zebrafish orthologues of TLR4 do not bind LPS (Sepulcre *et al.*, 2009, Sullivan *et al.*, 2009). Alternative receptors for LPS recognition in fish have been proposed, *e.g.* β 2-integrins (specifically CD11 and CD18) (Iliev *et al.*, 2005b), however the receptor(s) responsible for LPS recognition in fish is still unknown. The underlying mechanism behind LPS mediated alterations in neutrophil number and dynamic behaviour will be further discussed in chapter 4 of this thesis.

Live embryo infection models

Having determined that the bacterial component LPS can amplify acute inflammatory responses in embryos I next developed a live bacterial infection model with which to investigate complex inflammatory responses including the role of p38

MAPK in response to infection. It is known that infection with *M. marinum* via immersion of zebrafish embryos in medium containing bacteria or by caudal vein injection results in granuloma formation and disseminated disease (Davis *et al.*, 2002a). In pilot experiments in the laboratory using caudal vein injection infections there was a large degree of mortality due to aggregation of the bacteria within vessels. I therefore chose to deliver *M. marinum* via the yolk sac. I demonstrate that it is possible to introduce *M. marinum* via micro-injection into the yolk sac resulting in the spread of the bacterium throughout the embryo and leading to death. Using a dsRED expressing *M. marinum* it was possible to follow the spread of the mycobacterium from within the yolk sac throughout the tissues of the embryo. This corresponds to the findings of Clay *et al.* who reported that in zebrafish embryos disseminated disease was mediated by the migration of infected macrophages (Clay *et al.*, 2007). Macrophages anchor themselves to the underlying and overlying surface of the yolk sac in embryos at 2 dpf (Herbomel *et al.*, 1999) and it is likely that it is these macrophages are the first to encounter the mycobacterium at the yolk sac. Transgenic *zPu.1:EGFP* embryos were used in this infection model in order to determine whether or not macrophages in this tissue engulf the injected mycobacterium. The high level of expression of GFP in the yolk sac prevented successful imaging of this region. However by 48 hpi dual positive cells were visible outside, but close to, the yolk sac. Davis *et al.*, who inject bacteria directly into the blood stream, report macrophage uptake of mycobacterium as early as 1 hpi (Davis *et al.*, 2002a). It is likely that the different method of infection that I selected is the cause of the slower appearance of macrophages that have phagocytosed the fluorescent bacteria. Despite the apparent delay in uptake by of bacteria by macrophages, when compared to the caudal vein injection route, the rate of mortality I observed was faster (Davis *et al.*, 2002a). Where Davis *et al.* demonstrate 100% mortality at 9 dpi I demonstrate 100% mortality at 6 dpi following yolk sac injection. This acceleration in mortality perhaps reflects the injection of a greater number of bacterium in the initial injection. While the yolk sac may contain dissemination initially, by partitioning the bacteria away from the tissues, eventually when they become accessible to the macrophages they colonise the tissues rapidly. Alternatively it is possible that infected macrophages are slower to exit the yolk sac

disseminate throughout other tissues of the embryo as it is isolated spatially and by the yolk sac wall. However it is not possible to confirm this as Davis *et al.* do not provide the data required to compare the amount of bacteria introduced in the initial infection (Davis *et al.*, 2002a). Furthermore there may be differences in the amount of bacteria introduced that would effect survival.

Isolation of immune cells from adult tissue for functional and expression analysis

Wounding alone and injury in the presence of LPS results in the recruitment of low numbers of cells which prevents transcriptional analysis and investigation of the intra-cellular mechanisms that are activated in these quantitatively different types of recruitment. Similarly in the embryo infection model, whilst it is possible to investigate whole embryo transcriptional responses to mycobacterium infection (van der Sar *et al.*, 2009), the complex intracellular relationship within the mycobacterium containing macrophage may be lost against the background of the tissues making up the majority of the embryo. I therefore attempted to isolate myeloid cells from adult zebrafish with a view to stimulating these cells *in vitro* with LPS or mycobacteria for analysis of functional and transcriptional responses determined in an innate immune cell enriched population. It was possible to isolate and sort by flow cytometry all the main blood lineage types from the zebrafish adult WKM (Traver *et al.*, 2003b). I set out to isolate the myelomonocytes, and specifically neutrophil and macrophage populations in order that the functional and transcriptional responses of these individual cell types to LPS stimulation and *M. marinum* infection could be investigated. The flow cytometry profile produced by isolating the WKM cells was comparable with that of Traver *et al.* (Traver *et al.*, 2003b). Discreet populations sorted were defined as erythrocytes, lymphocytes, myelomonocytes and precursors according to the morphological phenotypic classification of Traver *et al.* (Traver *et al.*, 2003b). It was also possible to sort mononuclear phagocyte macrophage like cells by co-incubating WKM with GFP conjugated fluorescent beads prior to flow cytometric sorting of the cell suspension for uptake of beads. It was hoped that sorting WKM cells on phagocytic capacity would have allowed subsequent LPS

stimulation of *M. marinum* co-culture experiments with the aim of investigating the gene expression profiles in infected zebrafish macrophage like cells. However whilst the total number of cells sorted in the granulocyte containing myelomonocyte and the GFP positive populations was sufficient for cytopsin analysis of morphology, and in the case of myelomonocytes for LPS stimulation for morphological activation experiments (see Chapter 5 Figure 24), the quantity of RNA purified from the myelomonocyte and GFP positive sorted populations was insufficient for LPS/mycobacterium stimulation/phagocytosis assays and gene expression analysis experiments. It was also a consideration that the functional capacity and gene expression profiles of the cells may have been compromised by the prolonged time needed to prepare and sort the cells (> 3 hours). In a compromise that shortened the preparation procedure but sacrificed the purity of the cell types I performed LPS stimulation experiments on unsorted WKM cell suspensions and *in vivo* coelomic cavity stimulations (see Chapter 4 Figure 22 and Figure 26). The WKM cells suspensions contained mixed leukocyte cell types, it is established from the work of Traver *et al.* that a large proportion of these cells are myelomonocytes (24%) and 12% are neutrophils (Traver *et al.*, 2003b). Granulocytes make up 60-80% of the constitutive composition of cells isolated from the coelomic cavity, as established by Moss and colleagues. WKM cells were used for *in vitro* LPS stimulation experiments and the coelomic cavity for *in vivo* LPS stimulation experiments with cells lavaged, post stimulation, to determine transcriptional LPS mediated effects (see chapter 3). So whilst I have failed to isolate adequate numbers of viable purified neutrophil and macrophage cells I have developed models of zebrafish leukocyte stimulation experiments with which to analyse the effect of LPS treatment and the role of p38 MAPK.

Despite the number and quality of cells sorted from WKM being too low to perform stimulation or co-culture experiments there was sufficient RNA purified from the cells to perform quantitative expression analysis of cell markers of interest in order to characterise the sorted cell beyond morphological phenotype. Moss *et al.* (2009) performed non-quantitative PCR on cells derived from coelomic cavity by sorting based on forward and side scatter. Non- quantitative PCR is only capable of detecting the presence or absence. Here I determined that the relative expression of

the myeloid lineage cell markers *csflr*, *mpo*, and *pu.1* is in agreement with the cell types assigned by morphological phenotyping to cell types sorted from the WKM and mature cells derived from the coelomic cavity lavages. Macrophage specific *csflr* was expressed most highly in the WKM in the precursors subset. As *csflr* has been shown to be involved in the production of multiple myeloid cell lineages in zebrafish (Liongue *et al.*, 2009) and is known to be expressed by early myeloid precursor cell types in mouse (Tagoh *et al.*, 2002), the relative high expression of *csflr* in cells within this gate substantiates this designation of the cells in this gate as precursor cells. I have shown that the relative expression of *mpo* is far highest in the sorted myelomonocyte cell population of the WKM which confirms the results of Hall *et al.* who demonstrate *mpo* is expressed in WKM derived myelomonocytes (Hall *et al.*, 2009). Similarly the pan myeloid marker *pu.1* was found to have highest expression within the myeloid myelomonocyte cell population with lower expression in the precursor population and lowest expression in the lymphocyte population of the WKM, which confirms the specification of different cell populations within the WKM by morphological phenotype.

In the coelomic cavity derived cell fractions whilst expression of *csflr* was higher in the macrophage/granulocyte population than the lymphocyte containing population as might have been expected, equivalent expression of *csflr* transcript was also detected in the NCC and macrophage/granulocyte samples. Moss *et al.* also described expression of *csflr* in all three sorted cell populations although they do not compare relative expression of transcripts between populations (Moss *et al.*, 2009). Further to this the level of neutrophil specific *mpo* transcript is expressed 200 fold above the relative macrophage/granulocyte and lymphocyte containing populations strongly indicating that there are relatively more neutrophil cells within this population which differs from the claim that that these cells are predominantly NCCs derived from the lymphocyte lineage (Moss *et al.*, 2009). Neutrophils in fish species are known to have killing capacity and it has been demonstrated that the number of neutrophils contaminating leukocyte fractions correlates with the non-specific cytotoxicity of those cellular fractions (Sasaki *et al.*, 2002). Since Moss *et al.* attribute killing capacity to this NCC population, and here I find MPO most highly expressed in this population, it is possible that neutrophil contamination, and not the

presence NCC marker expressing cells, is responsible for the increased killing. Furthermore the killing capacity experiments performed by Moss and colleagues in which zebrafish coelomic cells were tested against NK cell targets used whole coelomic cavity cell fractions and not sorted fractions. They do not show definitively that the cells expressing NCC markers are responsible for the killing capacity. Further evidence of a contamination of myeloid cells within the NCCs fraction is provided with the relative high expression of the early myeloid marker *pu.1* in the macrophage/granulocyte and NCC populations versus the lymphocyte population.

The purpose of developing these different models of inflammation in zebrafish embryo and adult cellular assays was to produce systems in which the evaluation of the effects of p38 MAPK inhibition and Notch signalling defects on immune responses was possible. This aim has been achieved and the effect of inhibition of p38 MAPK in these model systems will be investigated in the following chapter.

Chapter 4. LPS induced modulation of leukocyte migration to wound injury is p38 MAPK dependent.

Aims and introduction:

Aims:

The principle aims of the experiments reported in this chapter were:

- To use the zebrafish models characterised in chapter three to investigate the role of p38 MAPK in inflammation.
- To use *in vivo* time lapse imaging in the tail injury model of cell recruitment (see chapter three) to develop computational single cell tracking in transgenic zebrafish embryos and to characterise the effect of LPS recruitment at the single cell level.

Introduction:

Having determined that LPS enhances the number of cells recruited to wound injury these experiments were extended to investigate potential effects of inhibiting p38 MAPK on leukocyte recruitment and migration in this system. In order to achieve this I developed, with my colleague Juliane Liepe who carried out the computational analyses of the biological data, a system of automated single cell tracking to determine the dynamical characteristics of single cell movement/migration to wound injury. Current models of single cell tracking *in vivo* either require invasive surgical procedures to gain access to tissues at high magnification or available non-invasive methods require specialized equipment, either multiphoton microscopes or MRI scanners working at high magnetic fields. Multiphoton imaging uses two-photon microscopy, which is capable of imaging living tissue up to a depth of one millimeter. It was first used to image

immunological interactions in 2002 (Bousso *et al.*, 2002, Miller *et al.*, 2002, Stoll *et al.*, 2002). These recent developments in real-time imaging analysis of the dynamics of single cells are advancing our knowledge of single cell interactions in the immune response. Miller *et al.* (Miller *et al.*, 2002) first used novel microscopic techniques to image single cell interactions in real time within explanted mouse lymph nodes. Antigenic challenge of T cells resulted in the switch from random walk movement to a “swarming” phenotype (Miller *et al.*, 2002). Dynamic cell imaging has been applied to demonstrate T-cell activation and migration following an antigenic stimulus (Stoll *et al.*, 2002). Advances following these initial studies, which imaged cell interaction in explanted tissue, allowed *in vivo* deep tissue imaging of cell-cell interactions (Halin *et al.*, 2005). Recently Rosenbaum *et al.* used video-microscopy to characterize T-cell and APC interaction in inflamed tissue for the first time using intravital microscopy in the iris (Rosenbaum *et al.*, 2008). The data acquisition step in this intravital imaging technique is limited by movements of the eye, including eye rolling and pupil contractions, as well as by movements caused by animal breathing and videos must be stabilized computationally after acquisition. Real time *in vivo* cell imaging is important in understanding cellular interactions within the immune system in order to gain understanding of disease processes.

Zebrafish and medaka embryos have been used in several studies to automatically track the migration of leukocytes to acute wounding *in vivo* (Grabher *et al.*, 2007, Redd *et al.*, 2006). These studies have used existing imaging software programs to design their tracking systems (Redd *et al.*, 2006, Grabher *et al.*, 2007) and do not correct for typical tracking errors as outlined by Beltman *et al.* in a recent review (Beltman *et al.*, 2009). Common artifacts include “switching” (cells overlap and it is difficult for the algorithm to determine one cell from the other when they separate), “double tracking” (algorithm can designate a long or stretched cell as two cells instead of one) and “border tracking” (cells near the edge of the field of view’s positions are estimated wrongly). “Switching” and “double tracking” errors can be corrected by manually viewing suspect tracks. “Border tracks” should be excluded from analysis (Beltman *et al.*, 2009). Here I report a novel automated cell tracking system that takes into account recent advances in the avoidance of cell tracking

errors and extends and improves upon previous models of automated cell detection and tracking *in vivo*.

Transparent transgenic zebrafish expressing GFP under leukocyte specific markers were used to produce time-lapse image sequences of the leukocyte response to acute injury. A system of computational automated cell tracking was designed with which the time-lapse biological data can be processed to analyse the dynamical migration of leukocytes to acute injury at the single cell level.

It has been reported that LPS can induce the expression of inflammatory cytokines (Watzke *et al.*, 2007) and mortality at high concentrations (Novoa *et al.*, 2009) in zebrafish embryos. LPS enhances human neutrophil migration via the activation of p38 MAPK (Aomatsu *et al.*, 2008) and I have demonstrated that LPS enhanced neutrophil recruitment to tail wounding in zebrafish embryos (Figure 4, Figure 5 & Figure 6 in chapter 3). LPS is also known to induce inflammation via the activation of p38 MAPK and in this chapter I investigate the role played by p38 MAPK in the LPS enhanced leukocyte migration to acute wound injury.

Here I describe an *in vivo* model that uses time-lapse imaging in conjunction with a novel automated mathematical method of cell detection to track and analyze leukocyte recruitment to damaged tissue. The effect of LPS stimulation on the number and directionality of recruited leukocytes is tested and to what extent any LPS mediated effects are dependent on the action of p38 MAPK was investigated.

Results

Development of automated single cell tracking system

Time lapse image acquisition of *zpu.1:EGFP* transgenic zebrafish embryos (which express EGFP under the myeloid specific Pu.1 promoter (Hsu *et al.*, 2004)) at 5 dpf was used to record the recruitment of leukocytes, *i.e.* PU.1⁺ cells, to a wound produced by tail transection. The development of a computational automated cell tracking and detection system (developed in collaboration with Julianne Liepe) allowed me to extract information from many films in a short time (Figure 14). The software takes into account typical tracking errors, recently documented by Beltman *et al.* (Beltman *et al.*, 2009), and minimizes them resulting in more reliable trajectory data. Figure 15 and Figure 16 are image sequences that demonstrate the ability of the automated single cell tracking system to detect multiple single cells (cells shown in red) within a wounded tail and track their movement over time (blue trajectory lines).

Leukocyte cell migration dynamics are spatially dependent

A time frame of 2-4 hpi was selected for analysis of dynamic behaviour as kinetic experiments (Figure 3 & Figure 18) had identified this period as optimal for cell recruitment. The kinetics of single cell migration to the injured zebrafish tail was captured using time-lapse microscopy and data processed to extract trajectory information for each leukocyte. I observed, from the extracted trajectory data, cells that performed either a directed movement or showed random walk characteristics. Several random walk aspects were analysed, including the directionality coefficient, isotropic behaviour and the distribution of projected step sizes onto the axis parallel to the wound (*X* direction) and parallel to the blood vessels (*Y* direction) (see material and methods for details). Cells that moved between the blood flow and the wound site showed a more directed movement than cells at the wound and cells more distant from the wound (Figure 17). This suggests a spatial-temporal gradient-

mediated dependency of leukocyte dynamics. Cells located between the blood flow and the wound displayed a biased random walk, i.e. they showed directed movement along the *Y*-axis but had a tendency to display random walk characteristics along the *X*-axis.

LPS enhances leukocyte recruitment and/or retention following tail transection in a p38 MAPK-dependent fashion

This experimental platform enables the screening of large numbers of embryos at the single cell level to determine the effect of modulators of cell migration. In chapter three I observed LPS enhanced the number of neutrophils recruited to acute injury (Figure 4, Figure 5 & Figure 6, chapter 3). Using the automated cell tracking system the effect of LPS on the level of immune cell recruitment was analysed. It was observed that the presence of bacterial components increased the number of cells observed at the site of injury. Immersion of embryos, wounded by tail transection, in LPS (10 µg/ml) increased the number of Pu.1⁺ cells found at the injury site. This effect was observed over the entire period of the experiment (Figure 18). Pu.1⁺ cell numbers around an un-stimulated injury site peaked at 5 hpw, the number of cells decreasing thereafter (Figure 18); these results are consistent with data from earlier experiments (Figure 3 & Figure 6) and those of other workers (Renshaw *et al.*, 2006). I next determined the effect of a pharmacological inhibitor of the p38 MAPK signalling pathway, SB203580, on the migration process. Although no effect of the inhibitor on the number of leukocytes at an un-stimulated wound site was observed, SB203580 completely abrogated the LPS-mediated enhancement of cell recruitment and retention (Figure 18). The temporal profile of cell migration remained identical across treatment groups, irrespective of the presence or absence of either LPS or SB203580, *i.e.* there was no observed delay in recruitment or shift in time of the peak in recruitment only an overall increase in cell number.

LPS-enhanced directionality of movement is p38 MAPK dependent

In the next set of experiments the influence of LPS on single cell migration dynamics was investigated. The directionality coefficient was calculated as well as the proportion of Pu.1⁺ cells showing a random walk for untreated and LPS treated wounds. The average directionality coefficient in response to LPS is 0.40 and is significantly higher compared to untreated groups (wound alone 0.30, +SB2030580 0.27, LPS + SB2030580 0.31), demonstrating that cells move in a more directed way through the extracellular matrix in the presence of LPS than an untreated wound (Figure 19 A). Random walk characteristics are suppressed in the LPS treatment response compared to the wound only (Figure 19 B-C). A more detailed study of the trajectories reveals that the changes are not equivalent on *X* and *Y*-directions (Figure 19 C-D). Even in the presence of LPS some leukocytes still perform a random walk in the direction parallel to the injury (*X*-direction), i.e. perpendicular to the target direction (Figure 19 D). The effect of LPS on the dynamics is reversed when the embryos are treated with SB2030580 (Figure 19). Note that an effect of LPS or SB2030580 on the average speed of the Pu.1⁺ cells (Figure 20) could not be detected.

LPS induces p38 MAPK dependent enhanced neutrophil recruitment to wound injury in a fixed embryo model

Having determined p38 MAPK dependent LPS enhancement of leukocyte cell number to tail transection injury over time in *zEGFP:Pu.1* transgenic embryos (Figure 16) I performed experiments to validate this effect in WT embryos and to test that the LPS effect was not limited to the *E. coli* strain of LPS. WT embryos were wounded by tail nick or tail transection and stained for the neutrophil marker MPO. Both *E. coli* and *S. enterica* derived LPS induced enhanced neutrophil recruitment/retention to wound injury in fixed and MPO stained WT embryos at 5 (dpf). Immersion of embryos wounded by either tail transection or tail nick injury in either *E.coli* or *S.enterica* LPS significantly enhanced neutrophil recruitment to an acute injury by 40% ($P<0.001$) and 50% ($P<0.001$) respectively in tail transection

injuries and 50% ($P<0.001$) and 65% ($P<0.05$) in tail nick injuries respectively (Figure 21 A-D). In the presence of SB203580 the *E. coli* LPS mediated enhancement of cell migration was reduced significantly to the level of that in the untreated sample for both the tail transection ($P<0.01$) and the tail nick injury models ($P<0.01$) (Figure 21 A-D). *Salmonella* LPS mediated enhancement of cell migration was reduced to levels of that in the untreated group in tail transection ($P<0.01$) and the tail nick injury wounds (Figure 21 A-D). SB203580 had no effect on the baseline levels of neutrophil recruitment in embryos with wound alone in both the tail transection and the tail nick injuries ($P>0.01$). These results measured in fixed and stained embryo tissue demonstrate that LPS from two different source organisms causes increases in recruitment/retention of neutrophils and is in agreement with the p38 MAPK dependent LPS enhancement in recruitment/retention seen in the live recruitment automated cell tracking system (Figure 18).

LPS induction of cytokine expression in coelomic cavity cells *in vivo* varies greatly between animals

As discussed in the previous chapter the low numbers of leukocytes recruited to wound sites in embryo injury models limits transcriptional analysis of the gene expression changes occurring in response to LPS and SB203580 at sites of injury. I have shown that it is possible to derive cells from coelomic cavity lavages and perform quantitative PCR to determine relative expression of lineage markers (Figure 13 chapter 3). Therefore using this *in vivo* coelomic cavity approach LPS stimulations were performed in order to determine transcriptional responses and to investigate the role of p38 MAPK in LPS induced changes in gene expression. p38 MAPK was first identified as a protein critical for production of the cytokines IL-1 and TNF in human monocytes in response to LPS (Lee *et al.*, 1994) and was subsequently shown to be required for LPS induced IL-10 expression in human macrophages (Ma *et al.*, 2001). The primary aim of these experiments was to confirm that in zebrafish, as in mammals, LPS induces the expression of cytokines via the action of p38 MAPK. Consequently zebrafish *tnfa*, *il-1*, and *il-10* genes were chosen for qPCR expression analysis in order to test the ability of LPS to induce

expression of genes predicted to be regulated by p38 MAPK. LPS induced cytokine expression in adult *in vivo* coelomic cavity cell stimulation experiments (Figure 22). Increases in the expression of *tnfa* (7, 8 and 42 fold), *il-1b* (6, 7 and 58 fold) and *il-10* (5, 24 and 10 fold) were observed in the coelomic cavity cells isolated from 3 different LPS stimulated adult fish (Figure 22). LPS stimulation of coelomic cavity cells *in vivo* induced expression of these cytokines in all fish tested, however the level of induction varied greatly between the three animals. There was variation in the fold induction induced by LPS stimulation between fish. This was most likely caused by the high level of genetic variation in laboratory strains of zebrafish which is known to be greater than the genetic variation of other laboratory strains of animals (Guryev *et al.*, 2006).

LPS induces expression of *tnf* in adult WKM but not embryo tissue

It was also possible to investigate the transcriptional response of zebrafish immune cells to LPS in adult zebrafish WKM derived cells. In this case by pooling WKM cells derived from a several adult fish before subjecting them to treatments *in vitro* it was possible to eliminate the inter-fish variation in LPS responsiveness observed in the coelomic cavity experiments. LPS induced *tnfa* expression in adult WKM derived cells in a dose dependent fashion with maximal induction of expression achieved at a concentration of 50 µg/ml, resulting in a 10 fold increase in transcript when compared to the untreated sample (Figure 23). Phorbol myristate acetate (PMA) was used as a positive control and increased *tnfa* transcript 70-fold (Figure 23).

Having determined that, as in mammals, zebrafish leukocytes respond to LPS stimulation with cytokine production, I then determined whether LPS induced responses were detectable in zebrafish embryos. The expression of *tnfa* in untreated embryo tissue relative to untreated adult WKM cells was tested. Relative to untreated embryos, resting adult WKM expressed higher (>300 fold) levels of *tnfa* transcript (Figure 23). Exposure of embryos to increasing concentrations of LPS (1 – 150

µg/ml) failed to induce *tnfa* expression, although gene expression was elevated (2 fold), albeit minimally in response to PMA treatment (Figure 23).

LPS mediated morphological activation of myeloid cells is absent in zebrafish

In order to compare the functional responses of zebrafish leukocytes to LPS stimulation with classical mammalian activation responses, and to confirm the biological activity of the *E. coli* LPS used in all stimulation experiments, I performed morphological activation assays *in vitro* on human neutrophils and zebrafish myeloid cells. Zebrafish sorted myelomonocytes (zMyelomonocytes), which are largely composed of granulocytes (Traver *et al.*, 2003a), stimulated with LPS showed no change in morphology whilst human neutrophils displayed activated cell morphology (adhesion and cell spreading with the formation of distinct lamellopodia). Both zebrafish myelomonocytes and human neutrophils displayed activated morphologies in response to stimulation with PMA, demonstrating the zMyelomonocytes did have the capacity for morphological activation (Figure 24).

SB203580 inhibits phosphorylation of p38 MAPK in zebrafish embryos

The amino acid sequence of the activation site of the p38 MAPK protein is conserved between zebrafish and mammals (Krens *et al.*, 2006). Consequently activated zebrafish p38 MAPK can be probed for using antibodies produced against the mammalian phosphorylated p38 (pp38). To determine the ability of p38 MAPK inhibitor SB203580 to modulate the activation of p38 in zebrafish, embryos were treated with LPS and SB203580 and the protein extracted for western blot analysis of p38 activation. Unstimulated WT embryos at 4 dpf have significant levels of activated pp38 protein (Figure 25 B and D). Stimulation of embryos with LPS did not increase detectable pp38 (Figure 25 B). Exposure of LPS stimulated embryos to both 10 and 100µM SB203580 resulted in a reduction in pp38 (Figure 25 B and D).

LPS induces cytokine expression in WKM cells *in vitro* via the action of p38 MAPK

LPS induced cytokine expression in adult WKM can be suppressed by the action of p38 MAPK inhibitor SB203580 (Figure 26). LPS induced a 10 fold increase in *tnfa* expression that was suppressed to levels of the DMSO treated sample in the presence of SB203580. Lymphotoxin alpha (LTA) is a member of the TNF family of ligands and receptors and was recently identified in zebrafish by Savan and colleagues (Savan *et al.*, 2005) and its gene expression is assayed here in parallel with *tnfa*. LPS induced a 10 fold increase in *lta* expression that was suppressed to a 4 fold induction on treatment with SB203580. LPS induced a 7 fold increase in *il1b* expression that was suppressed to a 2 fold induction treated sample in the presence of SB203580 (Figure 26). A PI stain was performed on WKM cells after four-hour incubation with DMSO or SB203580. The SB203580 treated sample showed a small increase (5-9%) in cell death when compared with the DMSO sample demonstrating that cell death was not the cause of the reduction in cytokine production.

LPS induces expression of chemokines via the action of p38 MAPK

Migration of cells through tissue requires the cells to follow chemotactic gradients. A recent genetic survey in zebrafish has revealed many potential orthologues of well-studied mammalian chemokines (Nomiya *et al.*, 2008) and I investigated whether the expression of any of these zebrafish chemokines was regulated by treatment with LPS or SB203580. Transcriptome profiling of zebrafish embryonic responses to salmonella infection revealed induction of expression of zebrafish *cxcl8* (*il-8*) and *cxcl-C1c* (Stockhammer *et al.*, 2009) . Several investigators have also shown that *il-8* expression/protein release is regulated by p38 MAPK (Bhattacharyya *et al.*, 2002, Ostrowska and Reiser, 2008, Li *et al.*, 2009, Marie *et al.*, 1999). The chemokine CXCL12 (also known as SDF-1) is known to be highly chemoattractive for lymphocytes (Bleul *et al.*, 1996) and the zebrafish orthologue of this gene *cxcl12a* is known to be expressed in embryo tissue (David *et al.*, 2002). For

these reasons I selected to assay the expression of the *il-8*, *cxcl-C1c* and *cxcl12a* chemokine genes in WKM cells treated with LPS and SB203580. LPS induced zebrafish *il-8* and *cxcl-C1c* chemokine expression in adult WKM cells resulting in 5 and 3 fold increases in transcripts, respectively. SB203580 alone had no effect on expression of *il-8* and *cxcl-C1c* when compared with the DMSO control samples. Treatment with LPS and SB203580 suppresses LPS induced expression of *il-8* by 40% and of *cxcl-C1c* by 60%. There was no change in the chemokine *cxcl12a* across all treatment groups (Figure 27).

Effect of SB203580 on infection with *M. marinum*

Since SB203580 has a functional effect on leukocyte migration, cytokine production and chemokine production in zebrafish I hypothesised it may have affected survival of embryos infected with *M. marinum*. Embryos were injected with PBS or *M. marinum*, as described in the materials and methods section, then immersed in either system water containing either DMSO or SB203580 and incubated for several days. To control for the effect of long-term immersion of embryos in SB203580 embryos injected with PBS alone were exposed to the inhibitor for several days. The long term exposure to the inhibitor proved toxic to the embryos and prevented further exploration of the effect of the inhibitor on *M. marinum* infected embryos (data not shown).

Figures

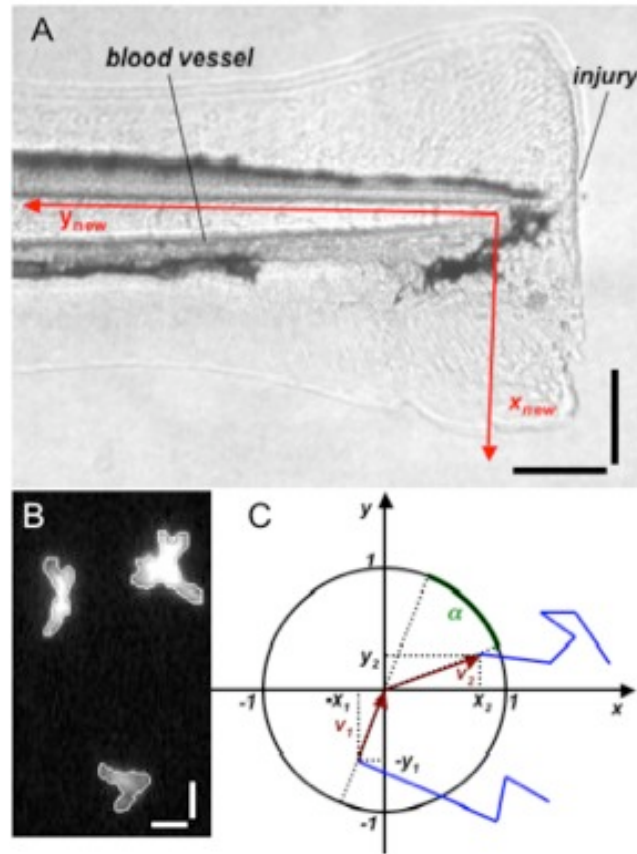


Figure 14. Bright field image of an injured zebrafish tail labels demonstrating data normalization and data analysis. In all generated movies the zebrafish were injured via tail transection so the injury is located orthogonal to the blood vessels. For analysis of data extracted from different movies they were normalized. Linear transformation of the trajectory data results into the new axes shown in red. The image resolution was constant in all movies. Scale bars are 100 μm (A). Microscopy image showing fluorescent Pu.1:eGFP leukocytes (B). The white line indicates the shape of the cells that have been detected using an automated image-processing algorithm and scale bars are 20 μm (B). An example trajectory (blue) would be tested for random walk characteristics. Two chosen motion vectors v_1 and v_2 (red) with their projections onto the x -axis and the y -axis (x_1, y_1 and x_2, y_2) are used to test for isotropy. Therefore the angle α between v_1 and v_2 was calculated. If the random walk model holds, the one-dimensional projections of the motion vectors onto the axes are Gaussian distributed (C).

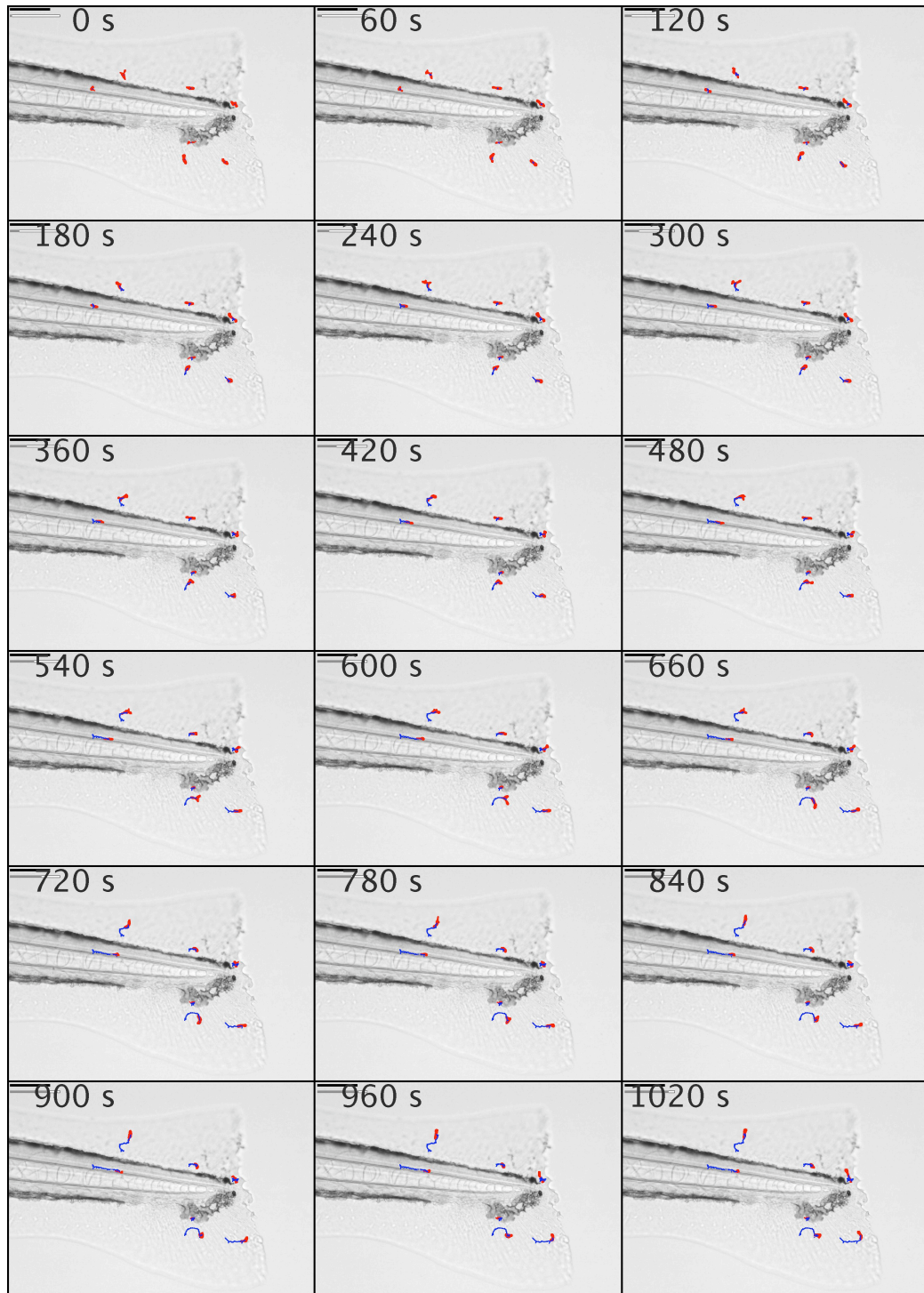


Figure 15. Time-lapse bright field images overlaid with images of Pu.1⁺ cells automatically detected (red cells) and tracked (blue trajectory lines) produced by automated single cell tracking system in a zebrafish *zPu.1eGFP* embryo wounded with tail transection. Time starts from 3 hpf and images shown are at 60 sec intervals (original time gap between images used for data analysis was 15 secs).

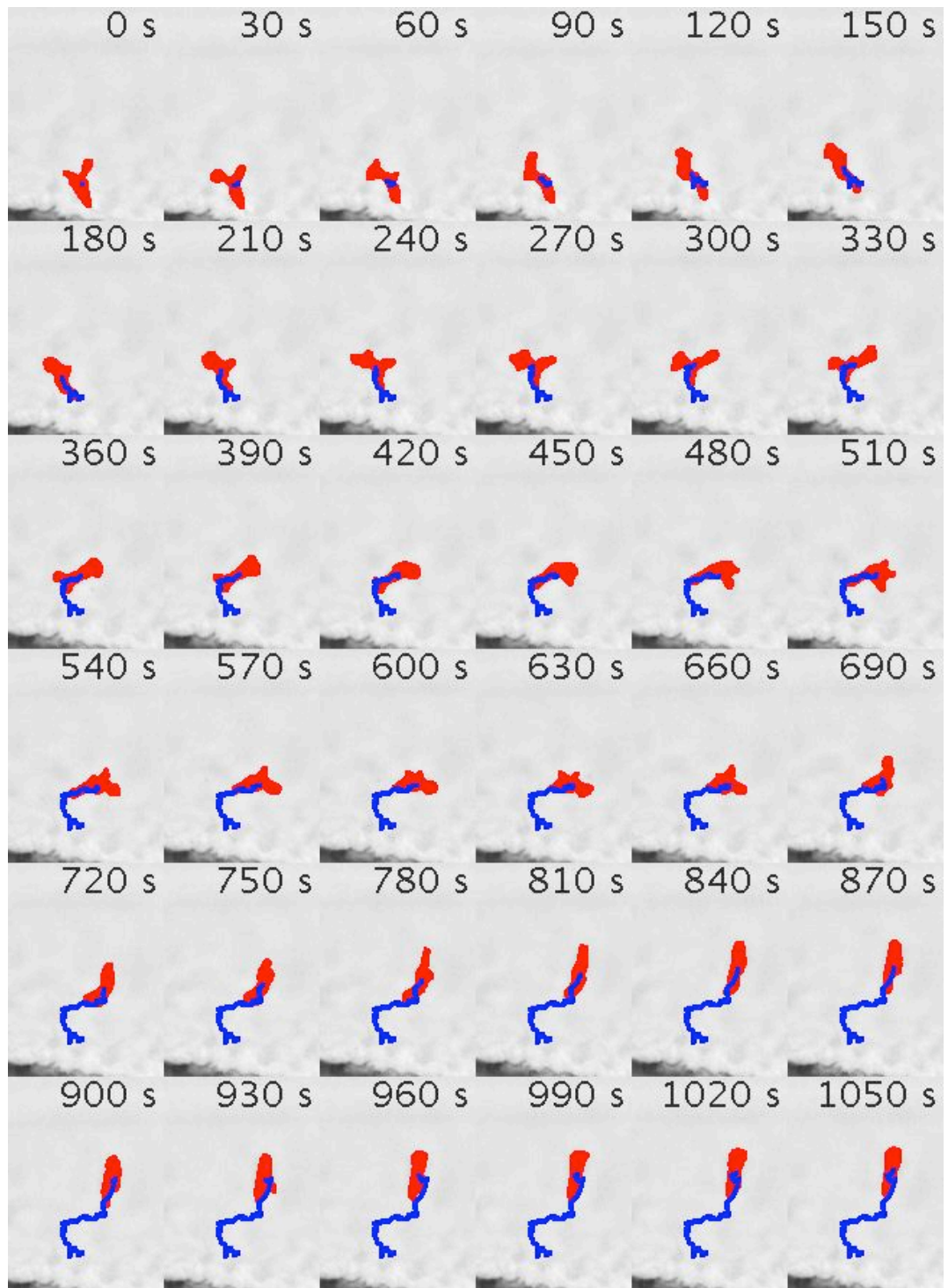


Figure 16. Time-lapse bright field images overlaid with images of single Pu.1⁺ cell automatically detected (red cell) and tracked (blue trajectory line) produced by automated single cell tracking system in a zebrafish *zPu.1eGFP* embryo wounded with tail transection. Time starts from 3 hpw and images shown are at 30 sec intervals (original time gap between images used for data analysis was 15 secs).

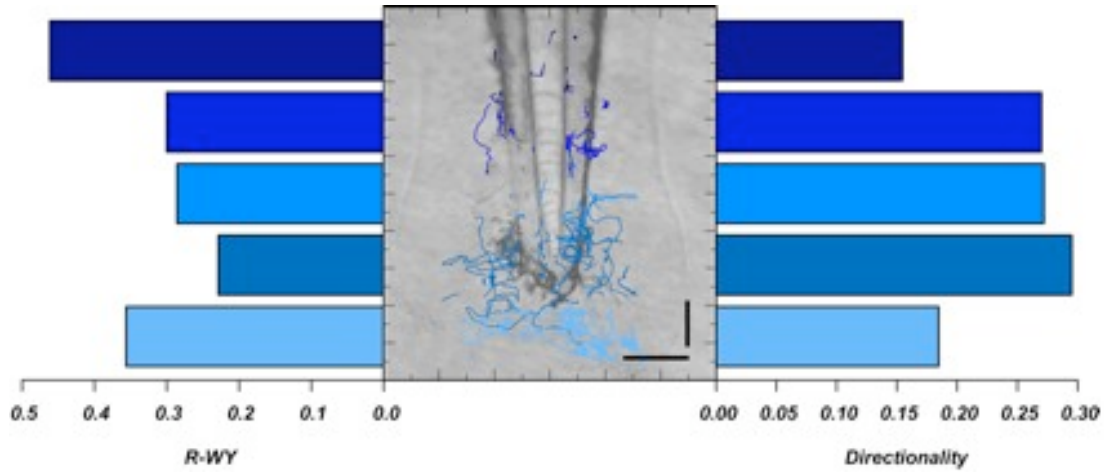


Figure 17. Leukocyte dynamical behaviour is dependent on the location of the cells in the organism. The detected trajectories extracted from all movies of untreated zebrafish are shown. Trajectories have been clustered according to their average distance to the wound in five groups and each of them is represented in a different colour. For each group the average directionality coefficient (right barplot) and the proportion of cells showing a random walk ($RW-Y$) (left barplot) is computed. Both dynamical properties show a dependence on the distance from the wound. Scale bars are 100 μm .

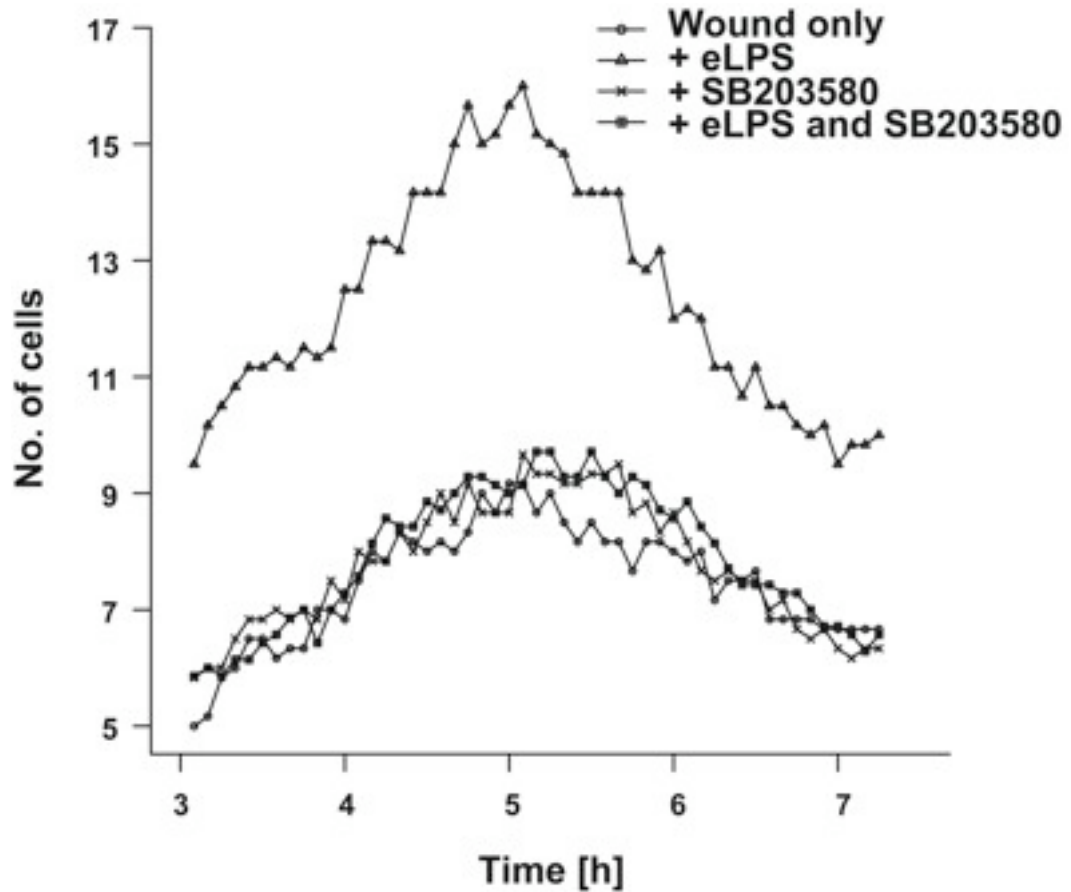


Figure 18. LPS treatment enhances recruitment of myeloid cells to a tail transection wound in *zpu.1:EGFP* zebrafish embryos (5 dpf). Embryos were wounded then immersed in system water or system water containing *E.coli* LPS (eLPS, 055:B5) at 10 $\mu\text{g}/\text{ml}$ in the absence or presence of SB203580 at 10 μM (+/- SB) and incubated for 2 hours before being transferred into agar and imaged microscopically. *zpu.1:EGFP* positive cells recruited to the wound were recorded at 5 minute intervals.

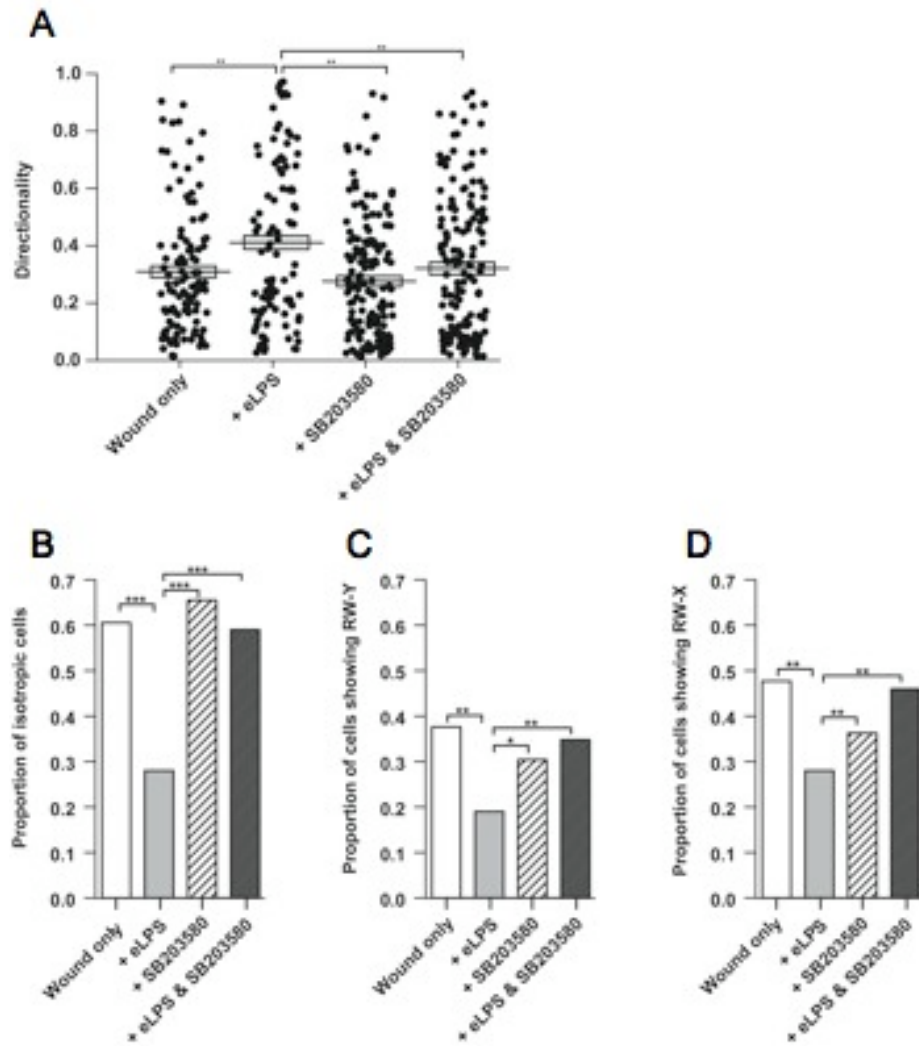


Figure 19. LPS enhanced directionality of cells can be ablated in the presence of p38 MAPK specific inhibitor SB203580. The directionality coefficients for each trajectory detected under one of the 4 described conditions. The lines indicate the expected values. The box indicates the 95% bootstrap confidence interval of the data distributions. The expected value of the directionality coefficient under LPS pressure is significantly higher compared to the remaining 3 conditions (A). Analysis of random walk (RW) aspects. The proportion of leukocytes that show one of the random walk aspects (isotropy of angles between two consecutive motion vectors is shown (B), Gaussian distribution of the projection of the motion vectors onto the y-axis (C) and x-axis (D), respectively) in respect to the total number of leukocytes detected (* represents $P > 0.05$, ** represents $P > 0.01$ and *** represents $P > 0.001$, computed with a randomized permutation test).

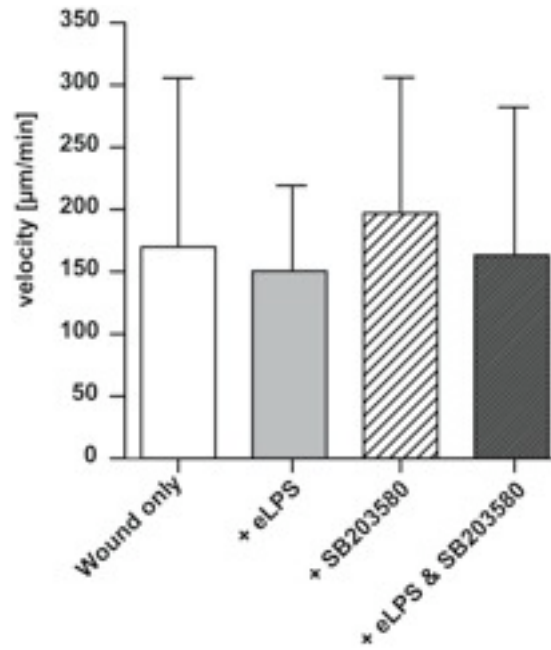


Figure 20. The average velocity of PU.1⁺ cell trajectories is not LPS dependent. *zPu.1:eGFP* embryos were wounded then immersed in *E.coli* LPS (eLPS) at 10 μg/ml in absence or presence of SB203580 at 10 μM (+/- SB203580). Error bars show the standard deviation. No significant differences were detected.

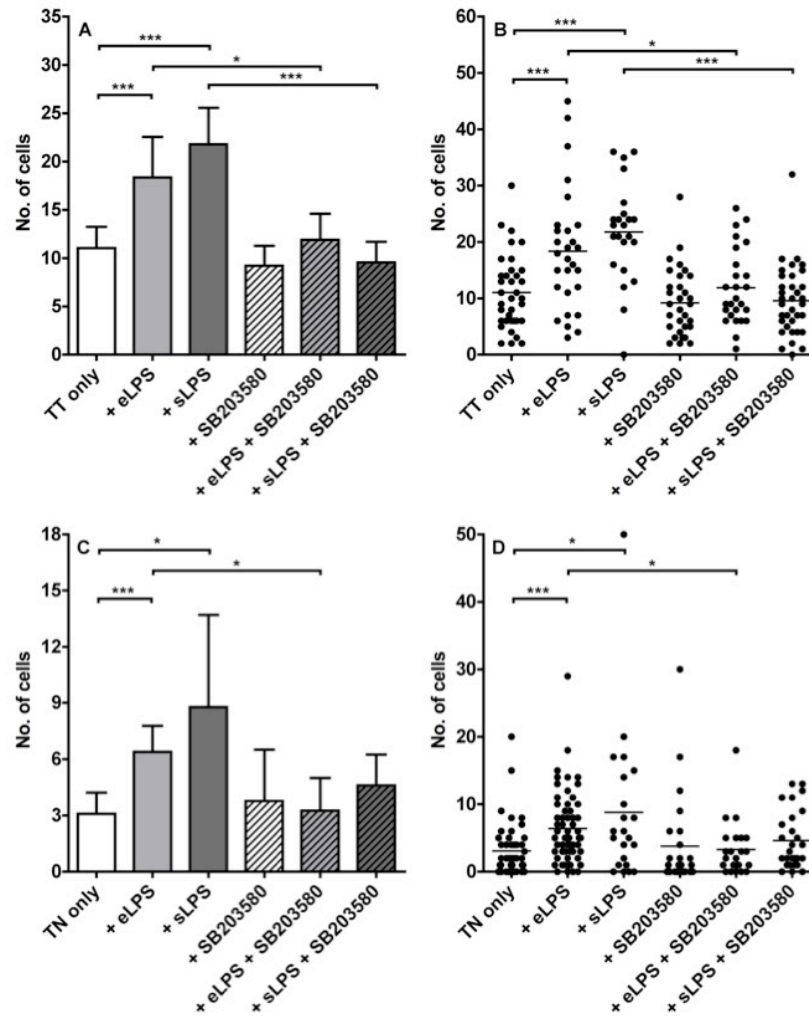


Figure 21. LPS treatment enhances recruitment of neutrophils to tail transection (TT, A & B) and tail nick (TN, C & D) wounds. Embryos at 5 dpf were wounded then immersed in *E.coli* (eLPS, 055:B5) or *S. enterica* (sLPS) LPS at 1 μ g/ml in absence or presence of SB203580 at 10 μ M (+/- SB) and at 4 hours (TT) or 2 hours (TN) post injury were killed, fixed and histochemically stained and scored for MPO positive cells at wound site (* represents $P>0.05$, ** represents $P>0.01$ and *** represents $P>0.001$). Bar graphs and dot plots represent the same data but showing individual data points for tail transection data (A & B) and tail nick data (C & D). Data were analysed using non-parametric one-way variance analysis (ANOVA) test with Dunn's multiple comparison post-hoc, Mann-Whitney test. Error bars shown are 95% confidence intervals. The experiments presented are representative of ≥ 3 performed.

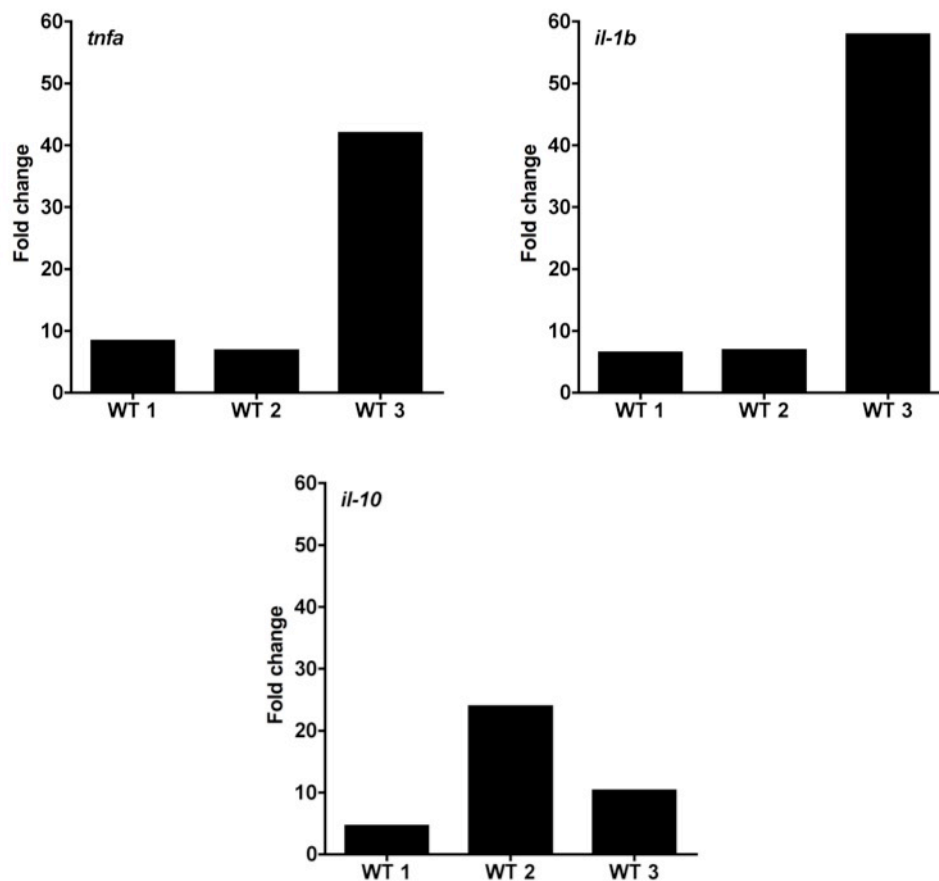


Figure 22. Quantitative PCR analysis of expression of zebrafish *tnfa*, *il-1b*, and *il-10* cytokine expression in adult coelomic cavity cells. Three adult WT fish had coelomic cavity flushed for cells at time zero and the cells lysed for RNA. LPS (10 µg/ml serotype 055:B5 in PBS) was injected into the coelomic cavity of each of the adult fish and after 4 hours coelomic cavity lavages were performed to isolate LPS stimulated cells which were lysed for RNA. RNA was processed to cDNA and taqman qPCR performed to assay gene expression. Gene expression is normalised to 18S and is presented as fold change relative to expression of the un-stimulated cells taken from each fish analysed. Bars represent expression of coelomic cavity cells from 1 animal.

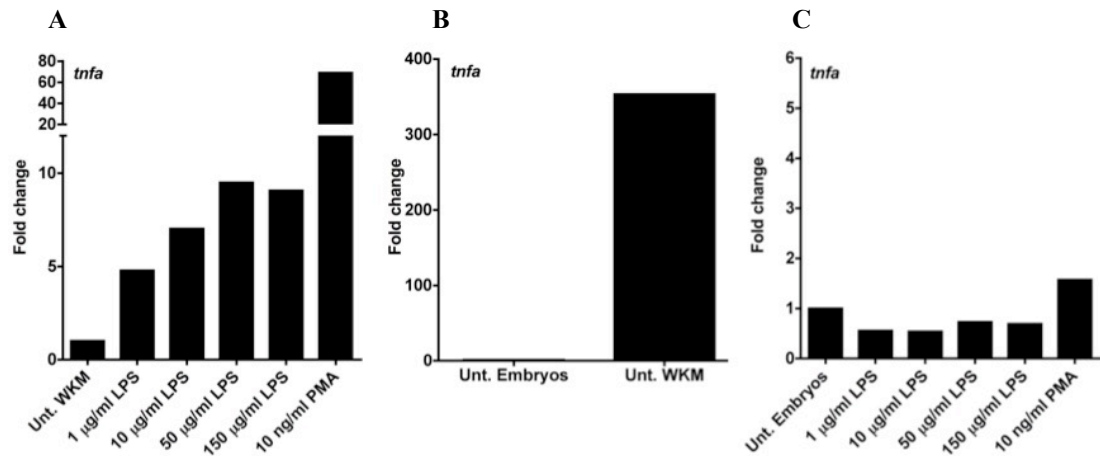


Figure 23. Quantitative PCR analysis of expression of zebrafish *tnfa* expression in adult WKM and embryo tissue. Stimulation with various concentrations of LPS (serotype 026:B6) or PMA (at 10ng/ml) was performed on WKM cells extracted and pooled from 10 adult fish and >25 embryos at 5 dpf and the tissues processed for cDNA. Gene expression is normalised to *efl-α* and is presented as relative to the mean of the unstimulated sample (A & C). Graphs are representative of ≥ 3 experiments performed.

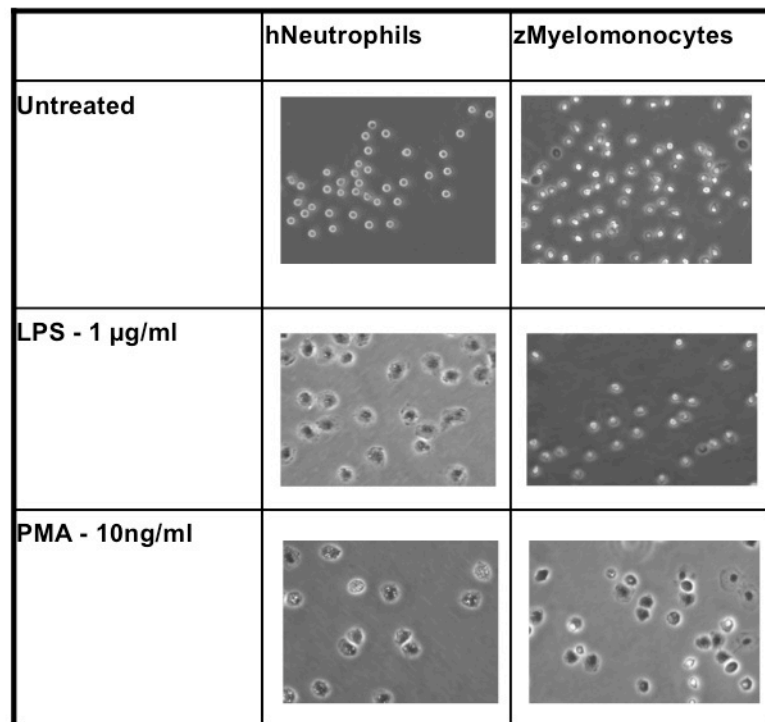


Figure 24. LPS induces change in cell shape of human neutrophils (hNeutrophils) but not zebrafish sorted myelomonocytic cells (zMyelomonocytes). Human neutrophils or zebrafish myelomonocytes were untreated or stimulated with *E.coli* LPS (serotype 055:B5, 1 µg/ml) or PMA (10 ng/ml) and visualised at 0.5 hrs. The images are representative of 3 experiments performed.

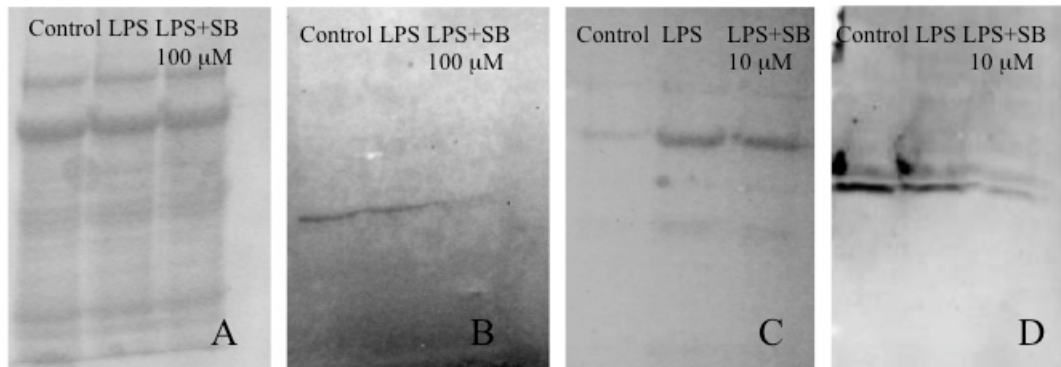
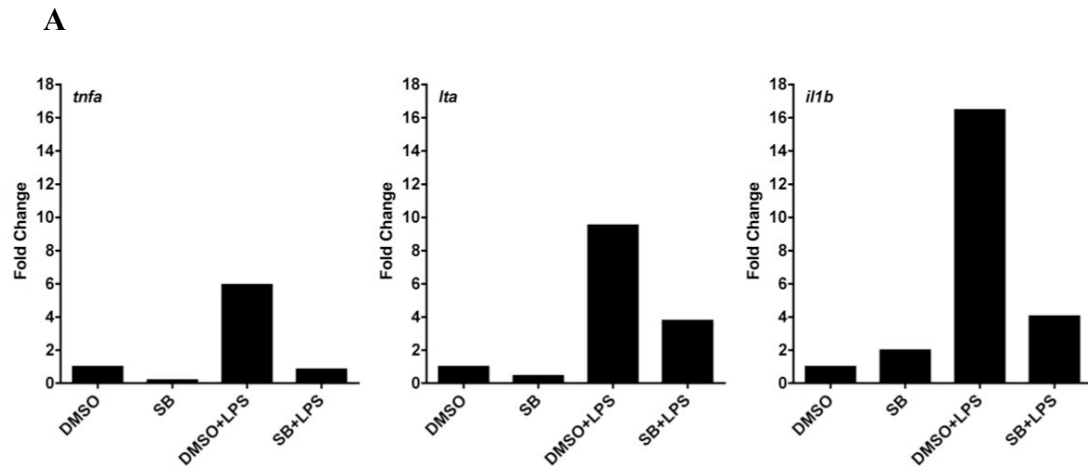


Figure 25. Western blots showing pp38 protein levels in 4 dpf embryos either untreated (control 1% DMSO), exposed to LPS (10 $\mu\text{g}/\text{ml}$) and LPS + SB203580 (at 10 or 100 μM as indicated) (B & D). The ponceau red stain for total protein is presented as a loading control (A & C).



B

	% cells FL-2 positive	
Treatment	WKM1 - PI	WKM2 + PI
DMSO	24.1	22.7
SB203580	33.8	27.5

Figure 26. Quantitative PCR analysis of expression of zebrafish cytokine expression in adult WKM tissue. WKM cells extracted from adult fish were stimulated with LPS (serotype 055:B5 at 10µg/ml) and/or SB203580 (10µM) and the tissues processed for cDNA. Gene expression is normalised to 18S and is presented as relative to the mean of the un-stimulated sample. Graphs represent expression of kidneys from one WT fish and are representative of at least 3 experiments performed (A). Table showing percent propidium iodide (PI) positive cells in WKM cells from two fish incubated with indicated conditions for 4 hours (B)

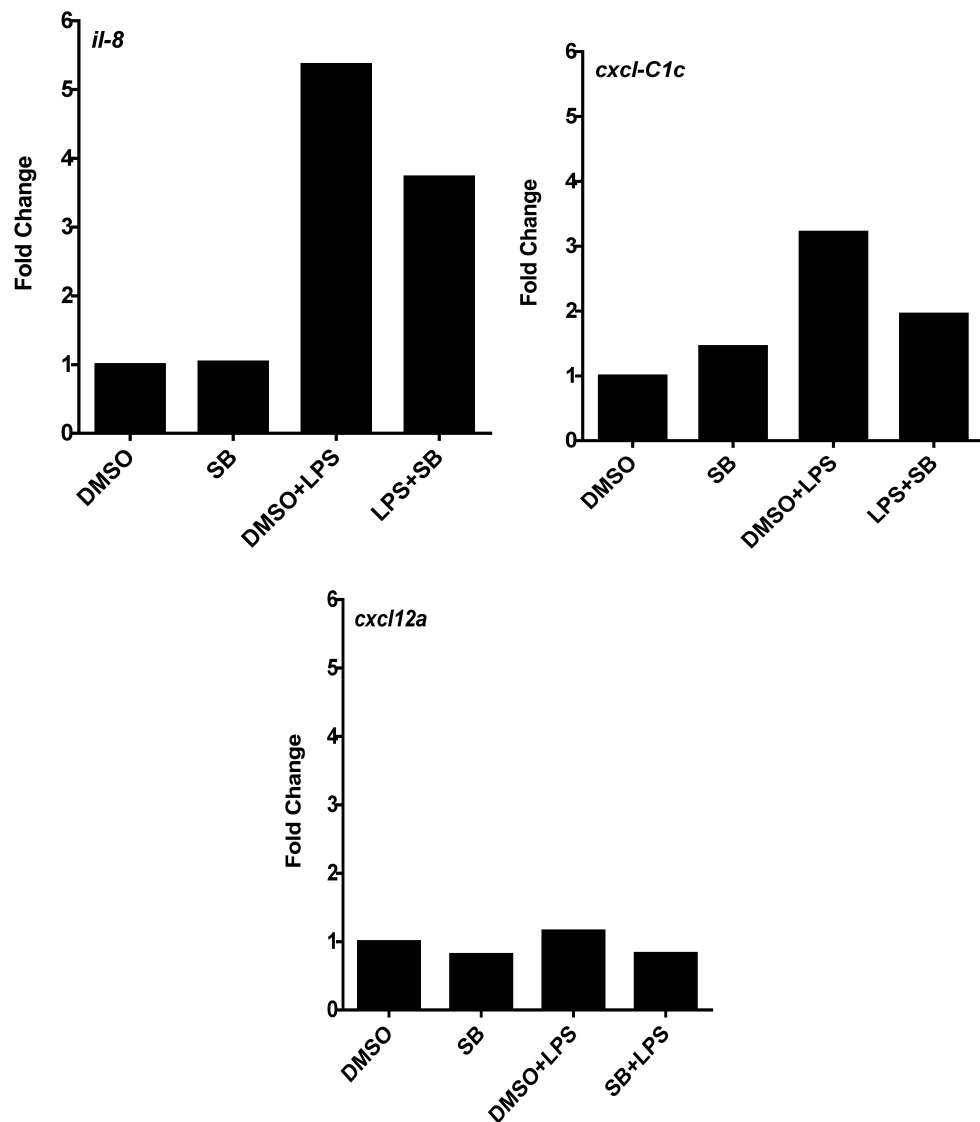


Figure 27. Quantitative PCR analysis of expression of zebrafish *il-8*, *cxcl-C1* and *cxcl12a* chemokine gene expression in adult WKM tissue. WKM cells extracted and pooled from adult fish were stimulated with LPS (serotype 055:B5 at 10 μ g/ml) and/or SB203580 (10 μ M) and the tissues processed for cDNA. Gene expression is normalised to 18S and is presented as relative to the mean of the un-stimulated sample. Graphs are representative of 3 experiments performed.

Discussion

In this chapter I have used data produced from time-lapse imaging of leukocyte recruitment to acute injury along with a novel computational system of automated single cell tracking injury to demonstrate LPS enhanced cell migration/retention behaviour is p38 MAPK dependent.

Leukocyte cell migration dynamics are spatially dependent

Those leukocytes located closest to the wound and those furthest away displayed similar random walk and directionality behaviour with these cells displaying a lower tendency towards directed movement and a higher tendency towards random movement at these positions. Cells located at an intermediate position, i.e. trafficking towards and away from the wound site, displayed more directional and less random behaviour. The observed decrease in random walk-like behaviour at intermediate distances might reflect the interplay between a signalling gradient and the leukocyte chemotactic machinery, which leads to directed movement towards the source of the signal. Niethammer *et al.* have reported that leukocyte migration during acute inflammation is dependent on the presence of an H_2O_2 gradient (Niethammer *et al.*, 2009). This gradient reaches up to 300 μm from the injured tail into the surrounding tissue and in this area the highest directionality coefficient was detected in leukocytes tracked. Once the leukocytes reached the injury and therefore the region of the highest H_2O_2 concentration, they exhibited “tumbling” behaviour. The increased number of trajectories showing a random walk behaviour located at greater distance to the wound could be explained by a low or absent H_2O_2 signal. Several cells migrated backwards from the wound towards the blood vessels, consistent with previous reports of retrograde leukocyte chemotaxis (Mathias *et al.*, 2006, Hall *et al.*, 2007). Whilst substantial progress has been made in understanding the complex molecular factors that orchestrate the movement of leukocytes from the vasculature into tissues less is known about the active movement of leukocytes back towards the vasculature. This migration of cells from the tissues

toward the vasculature is known as reverse chemotaxis and was recently reviewed by (Huttenlocher and Poznansky, 2008). It is reported that neutrophil chemoattraction or chemorepulsion behaviour on exposure to IL-8 (cytokine-induced neutrophil chemoattractant-1, CINC-1) is dependent on the concentration of IL-8 (Tharp *et al.*, 2006). Tharp and colleagues demonstrated *in vitro* (human neutrophils) and *in vivo* (rat neutrophils) that at low concentrations of IL-8 neutrophils were attracted and moved up the gradient of chemokine, however at increasing concentrations neutrophils changed behaviour and were repulsed, moved down the gradient (Tharp *et al.*, 2006). The mechanisms underlying bi-directional migration of neutrophils in zebrafish are not yet known and reverse chemotaxis of neutrophils in mammalian systems has not yet been clearly shown. Huttenlocher and colleagues propose two possible mechanisms for reverse migration of neutrophils in the zebrafish injury model, competing chemoattractive gradients or the contribution of both chemokinetic and chemorepulsive agents at the wound site (Huttenlocher and Poznansky, 2008). Here I have shown that the regulation of zebrafish *il-8* gene expression in zebrafish immune cells *in vitro* by LPS and the inhibitor SB203580 correlates with p38 MAPK dependent LPS enhanced migration of immune cells, suggesting a role for zebrafish IL-8 in the complex chemotactic control of neutrophil migration.

LPS enhances recruitment/retention and the dynamical behaviour of leukocytes in a p38 MAPK-dependent fashion

I have shown LPS increases the number of myeloid (Pu.1⁺ cells) (Figure 18) recruited and/or retained to an injury over time. There are more cells at the wound site in LPS treated embryos which is in agreement with the findings of Loynes *et al.* (2009) who report a reduction in apoptosis mediated by LPS (see Figure 28).

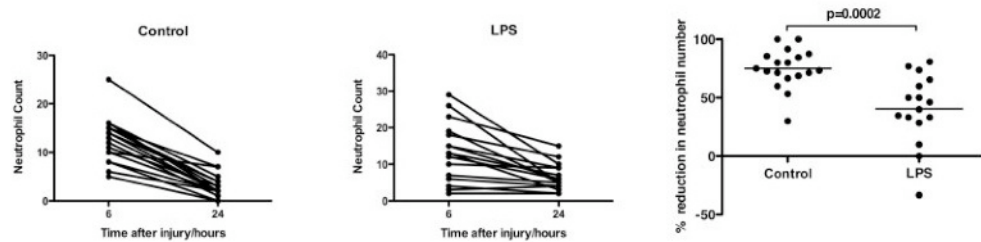
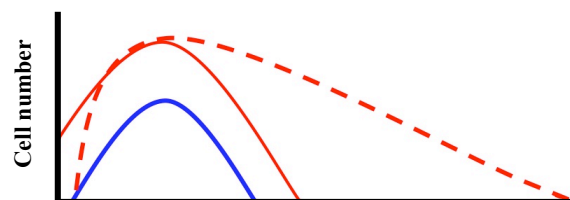


Figure 28. Adapted from Loynes *et al.* (2009). LPS (highly purified bacterial LPS 1 μ M Alexis Pharmaceuticals, San Diego, CA, USA) induces inhibition of neutrophil apoptosis which is associated with a defect in resolution of the cellular component of the inflammatory response following tissue injury in the zebrafish. Three dpf mpx:GFP zebrafish embryos were injured by tail transection and count of neutrophils at the sites of injury were made at 6 and 24 hpw. For each fish the percentage reduction in neutrophil numbers between these timepoints was calculated a measure of the percentage resolution of the neutrophil component of the inflammatory response.

However if delay/inhibition of apoptosis were the single cause of the observed increased cell number I would expect to observe a delay in resolution of recruitment leading to higher cell number over time (Loynes *et al.*, 2009). What is actually observed is an increase in cell number across the whole time course but no changes in the kinetic of the resolution of inflammation. This is perhaps best illustrated in a simple schematic (Schematic 8.).



Schematic 8. Schematic demonstrating the observed curves (Figure 18) – LPS (blue line) and + LPS (red line) and the predicted curve + LPS if inhibition of apoptosis alone were responsible for the increase in cell number (red dashed line).

If inhibition of apoptosis were the only factor responsible for the elevated number of cells at the wound a delay in resolution of recruitment would be expected as illustrated by the dashed red line (Schematic 8). However what is actually

observed is elevated numbers of cells across the whole time course but no change in the curve, no delay in the rate of resolution of the recruitment. Loynes *et al.* examined a longer and later time period after injury, it is possible that at later time points inhibition of apoptosis is the primary reason for elevated cells at the LPS treated wounds. During time period following the peak in resolution there is no delay in the resolution of the inflammation, and there are elevated cell numbers in the LPS injuries even before the peak in recruitment, suggesting it is necessary to look beyond cell number and consider cell behaviour to determine the underlying reasons for elevated cell number. Specifically the random walk and directional behaviour of these cells was analysed and it was found that LPS induced changes in the migratory behaviour of cells, which may explain the higher number of leukocytes observed at the wound site across the experimental period.

In order to determine the underlying mechanism of the elevated numbers of cells at the LPS exposed wound site the trajectory data for each single cell recruited was processed and measures of dynamic behaviour were quantified. This revealed that those cells exposed to LPS displayed a more directed movement and fewer cells displayed random walk behaviour when compared with untreated cells. This *in vivo* finding extends the observation of Aomatsu *et al.* who demonstrate *in vitro* that human neutrophils migrate in a random pattern and on LPS treatment display directed migration when the cells are further challenged with gradient concentrations of chemo-attractants (Aomatsu *et al.*, 2007). Furthermore, it has been reported that human granulocytes *in vitro* conform to random-walk theory and on addition of bacteria to these cultures they no longer do so (Peterson and Noble, 1972). Since we observe no increase in the speed of migration on treatment with LPS we can exclude the possibility that faster recruitment to the wound is responsible for the increase cell number at injury. The ability of LPS to induce more directed and less random behaviour in leukocytes provides an alternative explanation to inhibition of apoptosis as the underlying cause of higher cell number at wound site. If the tendency of cells towards directed movement is increased by LPS then it is reasonable to assume that more cells would find their way to the wound site resulting in higher cell counts over the course of the inflammatory response to injury.

I have shown not only that the LPS mediated increase in the number of neutrophil and PU.1⁺ cells recruited to injury but that this increase is p38 MAPK dependent. These *in vivo* findings extend the observations that increased migration of human neutrophils *in vitro* is induced by LPS activation via the action of p38 MAPK (Aomatsu *et al.*, 2007), and TNF α -primed human neutrophil migration to chemokine is p38 MAPK dependent (Montecucco *et al.*, 2008). The finding that the p38 MAPK inhibitor had no effect on the baseline levels of cell recruitment in fish with unstimulated wounds is consistent with observations of Zhang *et al.* (Zhang *et al.*, 2008), who find that migration of primitive zebrafish macrophages is mediated by JNK but not p38 MAPK, and suggests that there are two different pathways responsible for basal and LPS-induced recruitment. A hydrogen peroxide (H₂O₂) gradient is required for leukocyte recruitment to tail fin injury (Niethammer *et al.*, 2009) and since p38 MAPK is activated in the downstream signalling response to H₂O₂ stimulation (Guyton *et al.*, 1996), I might have expected that SB203580 would have had an inhibitory effect on recruitment in wound injury alone. However, this was not the case, suggesting that p38 MAPK is only required for the LPS-enhanced component of cell migration.

SB203580 as well as ablating LPS enhanced recruitment of cell number to injury was capable of ablating the LPS mediated effect on directionality and random walk behaviour. Hannigan *et al.* demonstrated that neutrophils deficient in Mitogen-Activated Protein Kinase-Activated Protein Kinase 2 (MAPKAPK2), which is a direct downstream target of p38 MAPK, showed a partial loss of directionality but an increase in migration speed (Hannigan *et al.*, 2001). Our results support a role for p38 MAPK in the shift of leukocytes to a directed movement however we observed no change in the speed of the migration on exposure to SB203580. Neither LPS nor SB203580 treatments had an effect on the speed with which the cells migrated suggesting that the larger inflammatory reaction results only from a larger number of recruited/retained cells, which migrate with increased directionality towards the injury. The suggested role for the p38 pathway in the *in vitro* study of Hannigan *et al.* study is supported by the finding of this study that the increase in the directionality coefficient of recruited leukocytes under LPS stimulation in live

embryos is p38 MAPK dependent. The *in vivo* results here go beyond these *in vitro* studies and demonstrate that leukocyte recruitment to an un-stimulated wound is unchanged by the inhibition of p38 MAPK but that the enhanced directionality and recruitment of these cells to the injury when challenged with the LPS is dependent on the action of p38 MAPK.

LPS induces expression of cytokines via the action of p38 MAPK

Here I report intra-coelomic injection of LPS resulted in induction of cytokine expression in coelomic exudate cells. This result extends the findings of Moss and colleagues who demonstrated that LPS injected into the coelomic cavities of adult zebrafish induced immune cell influx as it does in mammalian models of peritonitis (Moss *et al.*, 2009). However I noted there was considerable variation in level of induction of cytokine and contributing to this is the extensive genetic variation within laboratory strains of zebrafish which is markedly greater than that of other laboratory model organisms (Guryev *et al.*, 2006). In order to overcome this inter-animal variation in LPS responsiveness I performed *in vitro* LPS challenge experiments with WKM cells pooled from several animals.

Whilst other groups have reported LPS induction of *tnf* expression in embryo derived tissues here I did not observe this effect. Watzke *et al.* report induction of expression *tnf* in embryo tissue stimulated with concentrations as low as 1 ng/ml (Watzke *et al.*, 2007). This finding, that cytokine expression in a fish system at concentrations comparable with those that activate mammalian systems, disagrees with the findings of many research groups that show fish have a higher threshold of LPS sensitivity than mammals (Iliev *et al.*, 2005b). Others have demonstrated that much higher concentrations of LPS are required in order to induce *tnf* expression in embryos. Induction of expression of *tnf* was observed on exposure of TNF:GFP transgenic embryos to LPS but only at a very high concentration (140 µg/ml) (Yazawa *et al.*, 2005) which is only 10 µg/ml below the concentration lethal to embryos (150 µg/ml) (Novoa *et al.*, 2009). Oehlers *et al.* report only a 2 fold increase in *tnf* transcript on exposure of embryo derived intestinal epithelial cells to

LPS at 150 µg/ml (Oehlers *et al.*, 2010). In this study, even at the highest concentration tested (150 µg/ml), no increase in expression of *tnfa* was observed in the whole embryo. I have shown that LPS can induce a functional effect in embryos, enhancement of leukocyte migration, so it is possible that whilst LPS has a localised effect on activation of leukocytes, this effect is not detectable in whole embryos against the background of other tissues.

Iliev and colleagues report that the concentration of *E. coli* LPS required to induce expression of *tnf* in trout macrophages-like cells is 1000 fold higher (1 µg/ml) than the concentration required to activate mammalian macrophages. The maximal induction of *tnf* gene expression required concentrations that were orders of magnitude higher than the maximal concentration required for activation in mammalian systems (>10 µg/ml) (Iliev *et al.*, 2005a). Here I find maximal induction of *tnf* expression by *E. coli* LPS in zebrafish adult immune cells between 10 and 50 µg/ml in agreement with the findings of Iliev and colleagues. Consistent with this reduced sensitivity to LPS, when compared to mammalian responses, was the finding that whilst LPS activated human neutrophils morphologically, at concentrations capable of inducing *tnf* expression in WKM cells, the morphological activation phenotype of zebrafish myeloid cells by LPS was absent. These cells were capable of morphological activation, confirmed by activating with PMA as a positive control, demonstrating another example of lower LPS responsiveness in fish immune cells.

The inhibitor SB203580 has been shown to inhibit the phosphorylation of p38 MAPK and this is thought occur by inhibiting the ability of p38 MAPK to autophosphorylate (Ge *et al.*, 2002, Ashwell, 2006). Consistent with these findings in mammalian studies here zebrafish embryos exposed to SB205803 had reduced levels of pp38 when compared with DMSO treated controls, indicating that SB203580 inhibited the kinase activity of p38 as its ability to autophosphorylate inhibited in these embryos at the concentrations used.

Whilst it is not known which receptor(s) is responsible for LPS recognition in fish (Iliev *et al.*, 2005b, Sepulcre *et al.*, 2009, Sullivan *et al.*, 2009) this study and others, have shown that LPS is capable of inducing cytokine expression in fish leukocytes (Pelegrin *et al.*, 2001, MacKenzie *et al.*, 2003, Iliev *et al.*, 2005a, Sepulcre *et al.*, 2009). Sepulcre *et al.* (2009) also demonstrate activation NF-κB in

zebrafish embryos is independent of Myd88. In this study I add further insight into the signalling mechanism downstream of this unknown receptor and show that LPS induced expression of cytokine is dependent on signalling via p38 MAPK, as it is in mammalian systems (Lee *et al.*, 1994). This finding confirms the conserved function of p38 MAPK across species as an important mediator of inflammatory signals and supports the use of zebrafish as a model organism to study the pharmacological effects of inhibition of this protein.

LPS induces expression of chemokines via the action of p38 MAPK

In this study I have analysed the effect of LPS on the inflammatory behavioural responses of leukocytes as a population and at the single cell level in reaction to acute injury. However whilst this imaging and automated analysis approach permits interference in intracellular signalling mechanisms, by the use of pharmacological inhibitors, it was not possible to assay gene expression changes within the leukocytes, or in the whole embryo, to determine the underlying genetic control of the observed changes in inflammatory behaviour. For this reason I used adult WKM cells in *in vitro* stimulation assays to identify genes that are regulated by LPS in a p38 MAPK dependent way and therefore find candidate genes that may have a role in the behavioural changes observed. Using this method I have demonstrated p38 MAPK to have a conserved role in the mediation of the production of inflammatory cytokines. By choosing likely candidate chemokines I have demonstrated that zebrafish orthologues of *il-8* and *cxcl-C1c* are induced on stimulation with LPS and this induction can be attenuated using an inhibitor of p38 MAPK activity. LPS is known to induce expression of *il-8* in mammalian immune cells (Marie *et al.*, 1999, Mukaida, 1992, Harada *et al.*, 1994) and LPS induced expression of *il-8* is dependent on p38 MAPK signalling in some cell types (Marie *et al.*, 1999, Li *et al.*, 2009). Zebrafish *il-8* (*cxcl8*) is expressed on leukocytes and gene expression can be induced in epithelial cells by high dose stimulation (150 µg/ml) (Oehlers *et al.*, 2010). Here for the first time I have shown that zebrafish *il-8* can be induced by LPS in a p38 MAPK dependent manner, as it is in mammals. This *in vitro* gene expression data suggests that the regulation of the *il-8* and *cxcl-C1c* might

contribute to the complex migratory/behavioural effects mediated by LPS via p38 MAPK.

This study has shown experimentally that the *in vivo* migration of leukocytes exhibits spatio-temporally structured population heterogeneity. Furthermore, I have shown that LPS changes the recruitment, but does not alter *in vivo* leukocyte velocity. The observed effect of inhibiting p38 MAPK has two important implications: (i) it indicates that p38 MAPK is only involved in response to LPS (or challenge through pathogens) but not injury alone; (ii) it suggests that selectively targeting components of immune signalling pathways can change the overall behaviour in the leukocyte population. Due to population heterogeneity, I observe clear overlap in the chemotactic phenotypes. This heterogeneity, which was herein, demonstrated in a whole organism model system, will have clear implications for therapeutic interventions which may require attenuation of population heterogeneity in addition to a shift in the average behaviour as achieved here.

The novelty of this study lies in the large-scale comparative analysis of the characteristics, dynamics and signalling pathway dependency of leukocyte migration at the single cell level and the real-time *in vivo* response to wound injury in the presence and absence of endotoxin. The practical application of these findings lie in the potential use of this model to determine the efficacy of candidate therapeutics targeting innate immunity in conditions representative of un-stimulated and infected wound injury.

In this chapter I describe an *in vivo* model that uses time-lapse imaging in conjunction with a novel automated mathematical method of cell detection to track and analyze leukocyte recruitment to damaged tissue. I demonstrate that the number and directionality of recruited leukocytes is enhanced by LPS and show that this enhancement is dependent on the action of p38 MAPK.

CHAPTER 5. Deficiency in Notch signalling impairs myelomonocyte development resulting in diminished inflammatory responses.

Aims and introduction:

Aims

- In this chapter my aim was to investigate the effects of mutations in genes within the Notch signalling pathway on inflammatory responses and development of the haematopoietic compartment in larval and adult zebrafish.

Introduction

Whilst Notch signalling has long been known to influence cell fate and differentiation it is only in the last decade that its role in the immunological responses of fully differentiated immune cells has begun to be investigated. There is some controversy in the literature over interplay between the Notch and LPS mediated signalling. TLR induced responses, including those downstream of the LPS/TLR4 receptor complex, are regulated by components of the Notch pathway (Hu *et al.*, 2008, Monsalve *et al.*, 2009). LPS treatment has been shown to up-regulate Notch receptor expression and Notch signalling in macrophages via the action of p38 MAPK (Monsalve *et al.*, 2006). Conversely it was recently reported that LPS treatment results in the down-regulation of Notch signalling, for example down-regulating Notch1 and Hairy enhance of split (Hes1, a well characterised transcriptional target of Notch) expression, in mammalian macrophages (Kim *et al.*, 2008a) and microglial cells (Grandbarbe *et al.*, 2007). In this study I investigate the

effect of LPS treatment on WT and Notch mutant adult zebrafish immune cells in order to explore the regulation of LPS responses by components of the Notch pathway in an unbiased system, namely one in which mutations in Notch receptors and ligands are not directed to a specific cell type.

Notch signalling is critical in the development of many tissues and its contribution to haematopoiesis is now the focus of considerable interest. Notch receptors and their ligands are expressed on haematopoietic cells and play a role in haematopoietic stem cell (HSC) emergence, self-renewal, differentiation and lineage choices (e.g. Cheng *et al.*, 2003). The function of Notch genes in myeloid cell differentiation remains controversial with previous studies showing contradictory results highlighting the complexity of this pathway in development and differentiation. Constitutive expression of Notch1 through retroviral transfection of the intracellular domain (NiC) in 32D myeloid progenitors can inhibit granulocytic differentiation (Milner *et al.*, 1996). However Schroeder *et al* (Schroeder *et al.*, 2003) showed that transient activation of Notch1 signalling reduced the self renewal of multipotent progenitor cells and increased differentiation of granulocytes, macrophages and dendritic cell (DC) lineages through a direct increase in PU.1, a transcription factor involved in myeloid proliferation. Retroviral transduction of NiC in RAG^{-/-} mouse derived BM cells resulted in enhanced renewal of HSC, which favoured lymphoid as opposed to the myeloid lineage (Stier *et al.*, 2002). In contrast it was reported that transient Notch activation in irradiated adult zebrafish accelerated repopulation of all lineages without any skewing for lymphoid or myeloid lineage (Burns *et al.*, 2005). Notch deficiency in hematopoietic progenitor cells and Notch1 ^{-/-} embryonic stem cells resulted in impaired development of myeloid and lymphoid DCs in the mouse (Cheng *et al.*, 2003). Conditional deletion of Mindbomb in the mouse induced increased numbers of granulocyte progenitors leading to myeloproliferative disease (Kim *et al.*, 2008b) whereas in zebrafish, Mindbomb mutants analysed at 28 hpf revealed no differences in the numbers of cells expressing the myeloid markers PU.1, MPO and L-plastin as compared to wild type (WT) (Burns *et al.*, 2005).

Several zebrafish lines exist with mutations in genes encoding components of the Notch pathway. These mutants were identified from a large-scale ENU screen for

zygotic mutations that affected the embryonic development in zebrafish (Haffter *et al.*, 1996). Mutations in four Notch pathway related genes all resulted in abnormal somatogenesis (van Eeden *et al.*, 1996, Julich *et al.*, 2005). Two such mutants are *beamter* (BEA) and *deadly seven* (DES) that carry mutations in *delta C* and *notch 1a* genes respectively (Julich *et al.*, 2005, Holley *et al.*, 2002). These mutant zebrafish have developmental defects but are viable, probably due to gene duplication. The defects caused by the mutation in the *Notch1a* gene, characterised so far, include, perturbed motor axon outgrowth, neurogenesis, and somitogenesis (Gray *et al.*, 2001). Genetic manipulation of the Notch pathway in the mouse is hampered by the fact that in most cases the knockout of genes involved in this pathway is embryo lethal restricting this approach to conditional cell specific targeting of mutations. The availability of viable mutant zebrafish lines with defects in the Notch pathway provides a novel tool to investigate the function of this pathway in inflammatory responses and haematopoiesis.

Results

LPS mediated suppression of *hes1* gene expression is absent in DES mutant WKM cells.

In this study I have demonstrated that LPS induces cytokine and chemokine expression in zebrafish WKM cells. Since it has been reported that LPS stimulation of immune cells can result in suppression of signalling in the Notch pathway, I aimed to determine whether or not, as in some mammalian cell types, LPS would suppress Notch target genes in zebrafish immune cells. LPS stimulation of WKM cells in WT fish resulted in repression of the expression of *hes1* in a dose dependent manner. The highest suppression (50% when compared to the unstimulated sample) was observed at the highest concentration of LPS (10 µg/ml) (Figure 29 A). This suppression of *hes1* expression by LPS stimulation was not observed in WKM cells derived from DES mutants that have a mutation in the *Notch1a* gene (Figure 29 B). Inhibition of *hes1* specific transcripts was observed in the cells derived from WKM of BEA mutants (30% when compared to the unstimulated sample) at the highest concentration of LPS only (10 µg/ml), however this reduction in expression was not as marked as that observed in the WT (Figure 29 C).

Functional effects of mutations in the Notch pathway in zebrafish embryos

Since it is known that Notch has roles in the inflammatory response and myelopoiesis it seemed possible that zebrafish embryos with mutations in genes in the Notch pathway might display defects in recruitment of cells to a site of acute inflammation. In order to test this hypothesis I compared the number of neutrophils that were recruited in tail transection injury in WT and Notch mutant embryos. A significantly lower number of neutrophils were recruited to the wound site in BEA (10 cells vs. 8 cells, $P < 0.05$) and this was more marked in the DES mutants (10 cells

vs. 4 cells, $P < 0.001$) (Figure 30). While this experiment demonstrates that there is indeed a reduction in the number of neutrophils recruited to acute injury in Notch mutant embryos it does not identify the mechanism(s) that underlie the defect. This reduction could be due to a Notch mutation mediated defect in myelopoiesis with fewer neutrophils developing in the embryo resulting in fewer neutrophils available for recruitment, or a functional defect restricting the ability of the neutrophils to migrate towards the wound or a combination of both.

To assess whether the low recruitment in the mutants was due to an impaired migratory function of these cells in the absence of Notch signalling WT embryos were treated with the γ -secretase inhibitor, DAPT (50 μ M), which is effective in inhibiting Notch signalling in zebrafish embryos at this concentration (Geling *et al.*, 2002). Inhibiting Notch signalling shortly before and throughout the acute neutrophil response did not affect the number of cells migrating to the wound site in WT fish (Figure 31 A). Confirmation that DAPT treatment was effective was obtained by demonstrating that *hes1* mRNA was reduced in treated embryos as compared to untreated fish or DMSO vehicle treated controls (Figure 31 B).

In order to further confirm that defects in Notch pathway signalling is correlated with a reduction in neutrophil recruitment expression of the Notch target gene zebrafish *hes1* expression was determined in WT, BEA and DES embryo derived tissue samples. Expression of *hes1* in embryonic tissue was significantly lower ($P < 0.01$) in DES mutants when compared with WT tissue (Figure 32). BEA embryo tissue had comparable levels of expression with WT tissue (Figure 32).

Deficiencies in Notch signalling impairs myelomonocyte development

Defects in recruitment of neutrophils to acute injury in BEA and DES embryos was not mimicked using the Notch signalling inhibitor DAPT. This observation suggests that there may be a developmental defect in haematopoiesis in the myeloid compartment and to investigate this possibility flow cytometry was performed on WKM immune cells derived from adult WT and mutant zebrafish. Results of the flow cytometry experiments performed on 2-3 month old adult mutant and WT fish using cells from the WKM (Figure 33 A) showed that the percentage of

myelomonocytes was significantly lower in Notch mutants BEA ($p<0.001$) and DES ($p<0.01$) compared to WT while percentages of lymphocytic cells were higher in mutants (BEA ($p<0.01$) and DES ($p<0.01$) vs. WT) (Figure 33 B and C). The percentage of precursor cells was similar in all groups (Figure 33 D). The total number of cells collected from WKM was similar in all fish indicating that Notch mutants have lower overall numbers of myelomonocytes than WT fish.

This trend was reflected in the periphery with a significantly lower percentage of granulocytic cells in the coelomic cavity of mutants when compared with WT (BEA ($p<0.05$) and DES ($p<0.01$) vs. WT) (Figure 34 B). Again the lymphocyte containing populations showed higher percentages in mutants compared with WT (BEA ($p<0.05$) and DES ($p<0.05$) while no significant differences were observed in the nonspecific cytotoxic cells (NCCs) described by Moss *et al* (Figure 34 C & D).

Defect in myelopoiesis has no effect on survival in *M. marinum* infection experiments

Here I report that mutations in zebrafish *notch1a* and *deltaC* genes results in the development of fewer myeloid cells in adult and embryo. To further explore the potential differences in host cellular innate responses in the Notch mutant WT and DES embryos (which showed the strongest reduction in neutrophil recruitment phenotype in the embryo injury model) were infected with *M. marinum*. DES embryos were chosen as they showed highest differences in cell proportions when compared to WT. Survival time in response to bacterial challenge was equivalent between WT and DES embryos (Figure 35).

Figures

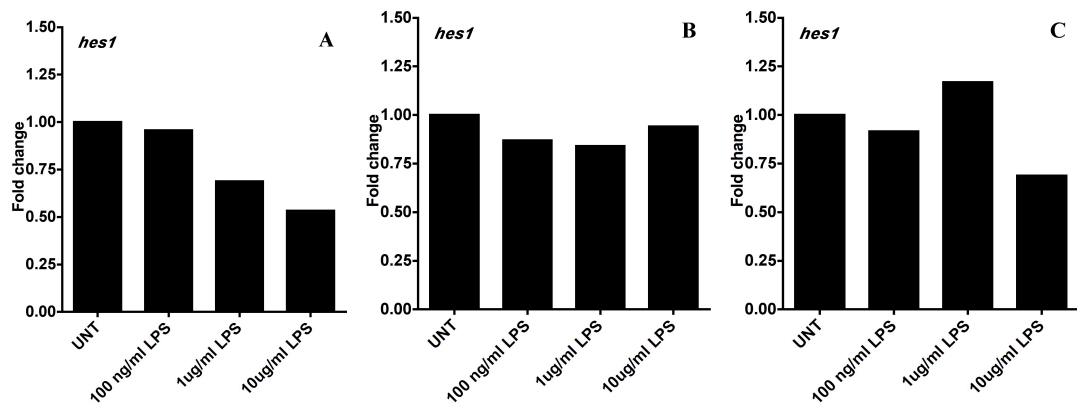


Figure 29. Quantitative PCR analysis of expression of zebrafish *hes1* expression in adult WT (A), DES (B) & BEA (C) WKM tissue. Stimulation with various concentrations of LPS (serotype 055:B5) was performed on pooled WKM cells extracted from adult fish and the tissues processed for cDNA. Gene expression is normalised to 18S and is presented as relative to the mean of the unstimulated sample, results of 1 experiment representative of ≥ 3 performed.

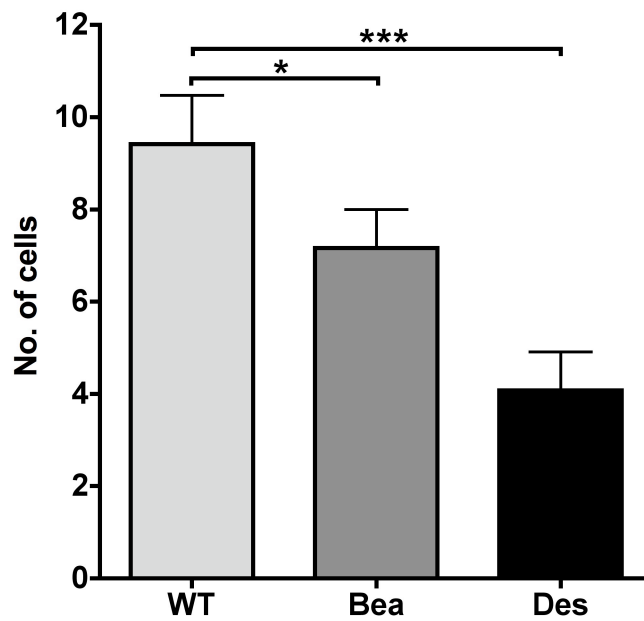


Figure 30. Reduced recruitment of MPO stained cells to a tail transection wound injury in 5dpf BEA and DES mutant embryos compared to WT (A). 5 dpf zebrafish embryos were submitted to a tail transection and after 4 hours stained for MPO. MPO positive cells recruited to the wound site were counted and groups compared as follows, WT (light grey bars), BEA (dark grey bars) and DES (black bars). (D). The data presented are representative of at least 3 experiments.

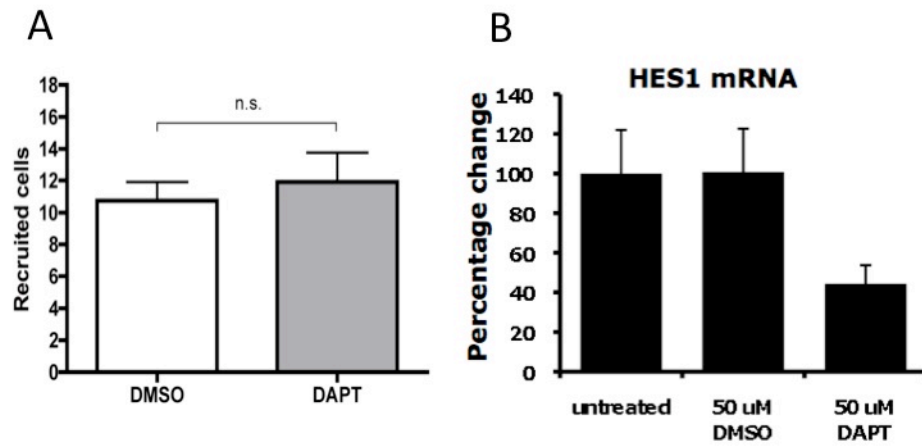


Figure 31. 5 dpf WT embryos were treated with DAPT (50 μ M) or with equivalent levels of DMSO control for 4 hours prior to being submitted to a tail transection. After 4 hours fish were fixed and stained for MPO. MPO positive cells recruited to the wound site were counted and groups compared as follow, DMSO (white bars), DAPT (light grey bars) (A). WT embryos were collected following DAPT and DMSO treatment and RNA extracted for analysis of Hes1 mRNA levels by TaqMan RT-PCR, results were normalised to 18S and relative expression compared to untreated controls (B). The experiments shown are representative of at least 3 performed.

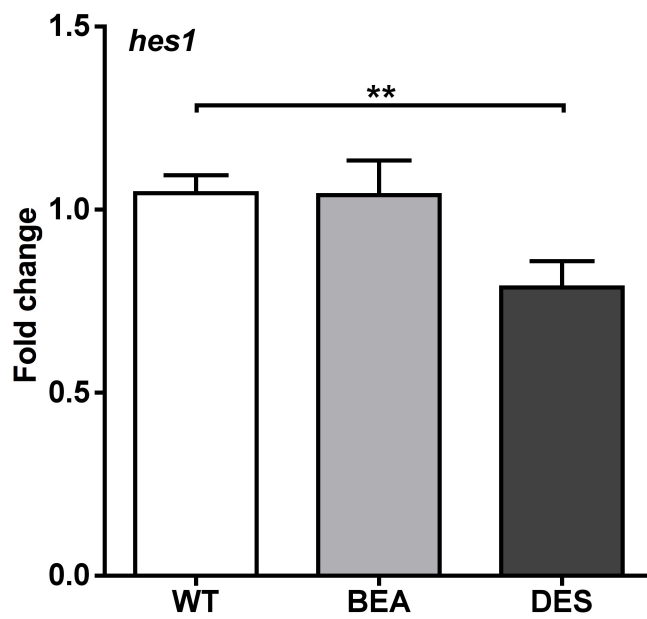


Figure 32. Quantitative PCR analysis of expression of zebrafish *hes1* expression in WT and Notch mutant embryo tissue (4 & 5 dpf). RNA lysates from WT fish (white bar), BEA (grey bar) and DES mutant fish (black bar) and processed for cDNA. Gene expression is normalised to 18S and is presented as relative to the mean of the unstimulated WT sample. Bars represent mean expression from 5 separate pooled samples. Embryo tissue lysates were produced with >30 embryos in each pool.

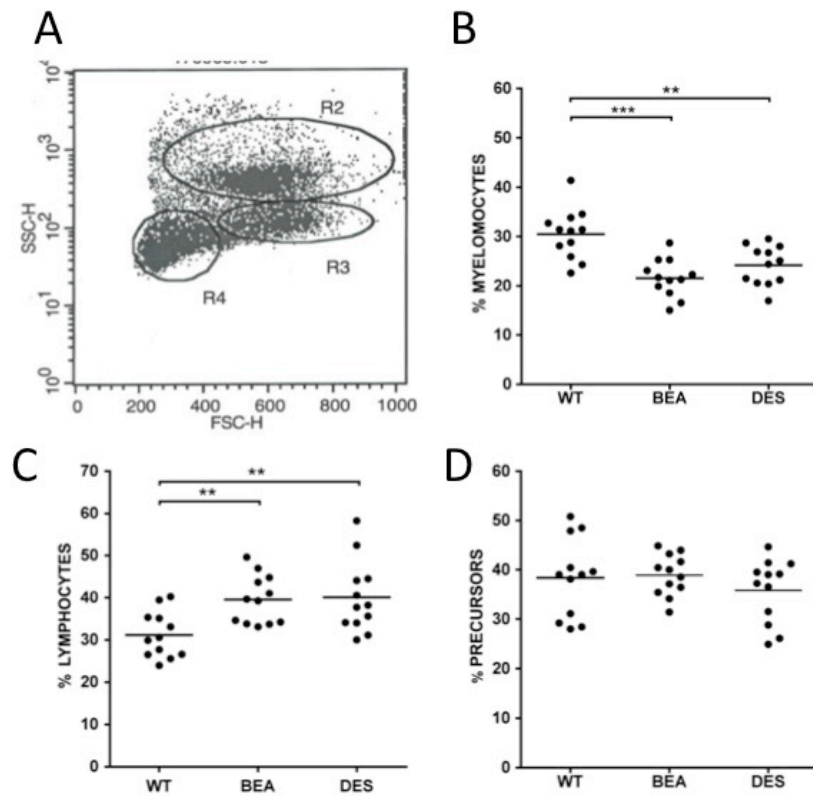


Figure 33. Leukocyte proportions in WKM are affected in Notch mutants. Cells isolated from WKM of WT, BEA and DES mutants were obtained from adult zebrafish and analysed using flow cytometry based on their forward and side scatter characteristics. A typical profile of live cells obtained for WKM (A). The gates used for the analysis of various cell populations were as follow R2= myelomonocytes, R3= precursors and R4 lymphocytes. Percentage of gated cells were plotted for each fish (B-D). Data are presented with line at mean and analysed using one-way variance analysis (ANOVA) test with Bonferroni's Multiple Comparison.

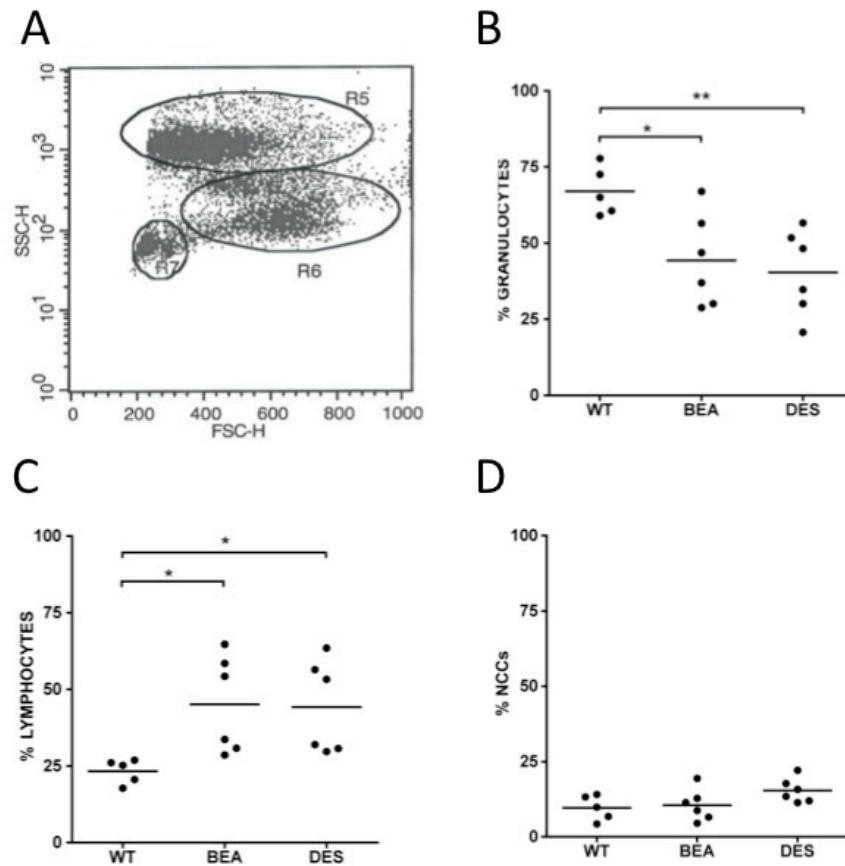


Figure 34. Leukocyte proportions in coelomic cavity are affected in Notch mutants. Cells isolated from CC of WT, BEA and DES mutants were obtained from adult zebrafish and analysed using flow cytometry based on their forward and side scatter characteristics. A shows a typical profile of live cells obtained for CC. The gates used for the analysis of various cell populations were as follows R5=granulocytes, R6=lymphocytes, R7=NCC for Coelomic cavity. Percentage of gated cells were plotted for each fish (C-E in WKM and F-G in CC). Data are presented with line at mean and analysed using one-way variance analysis (ANOVA) test with Bonferroni's Multiple Comparison.

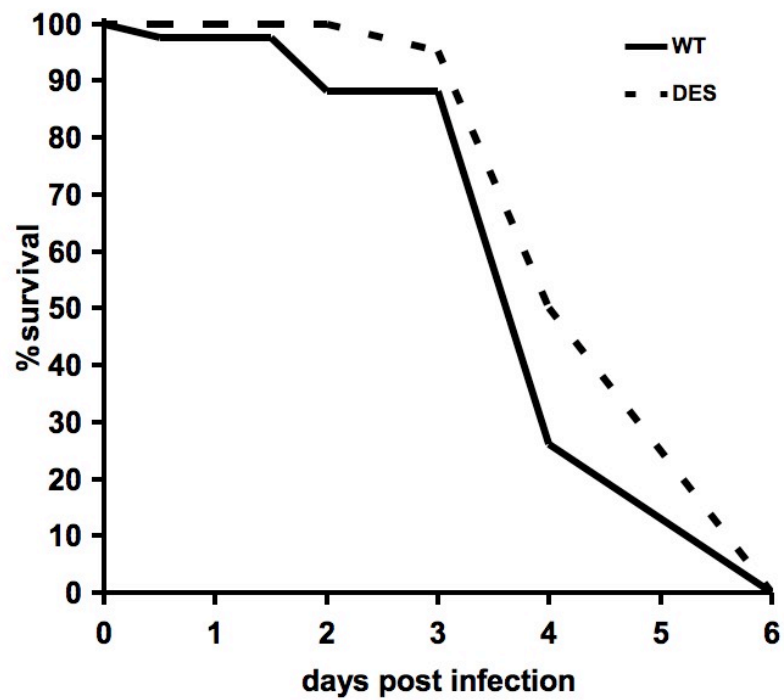


Figure 35. Survival following infection with *M. marinum* is unaffected in Notch mutant embryos. WT and DES mutant embryos (over 50 in each group) were injected at 28 hpf with dsRed *M. marinum*. Survival was monitored daily and survival curves are shown for each groups. The experiments shown are representative of at least 3 performed.

Discussion

LPS mediated suppression of *hes1* gene expression is absent in DES mutant WKM cells.

There is controversy regarding the effect of LPS on the expression of Hes1, a gene transcriptionally regulated by Notch signalling. Several research groups have reported LPS activation of various cell types resulted in increased Notch1 transcription and protein levels (Li *et al.*, 2001, Monsalve *et al.*, 2006, Monsalve *et al.*, 2009). This upregulation of Notch might be expected to result in the subsequent upregulation of Hes1, a target gene of Notch signalling. Indeed it has been described that Hes1 expression increased in response to LPS exposure in a macrophage cell line (Palaga *et al.*, 2008) and in human primary human macrophages (Hu *et al.*, 2008). In contrast Kim *et al.* showed Hes1 protein and mRNA gene expression levels were suppressed after LPS exposure via the Notch signalling pathway in the same human macrophage cell line used by Palaga and colleagues (Kim *et al.*, 2008a, Palaga *et al.*, 2008). It was also found that LPS treatment of primary rat microglial cells resulted in downregulation of the expression of Hes1 (Grandbarbe *et al.*, 2007). Here I show zebrafish *hes1* gene expression is suppressed in a dose dependent manner in adult zebrafish immune cells, which would support the studies that identify LPS as a suppressor of Hes1 expression. This suppression was absent in WKM cells derived from adult DES (*Notch1a*) mutant fish which confirms the LPS induced *hes1* suppression in zebrafish immune cell responses is Notch dependent. The weaker suppression of *hes1* expression observed in the BEA fish can be explained by the fact that these fish have a mutation in only one ligand of the Notch ligand family, of which there are several, and so a compensatory mechanism involving the remaining functioning ligands may counter balance the effect of the loss of function of single ligand. Results from our laboratory show that bone marrow derived dendritic cells (BMDCs) stimulated with a combination of LPS and Jagged respond with a downregulation of Hes1 gene expression (Bugeon *et al.*, 2008 and

personal communication with Dr M Gentle). This finding would also support the published studies that reveal LPS to be a suppressor of Hes1 expression.

Functional effects of mutations in the Notch pathway in zebrafish

Here I have shown BEA and DES zebrafish with mutations in Notch pathway gene exhibit defects in myelopoiesis from an early stage of development and into adulthood. BEA fish with mutations in the *DeltaC* gene displayed a weaker phenotype than the DES fish, which have mutations in the *Notch1a* gene. For example injury recruitment, suppression of expression of *hes1*, and the proportion of granulocytes in the coelomic cavity were more pronounced phenotypes in the DES mutants. This is perhaps explained by the fact that the mutation in the DES fish is in receptor whereas the BEA mutants have defects in the ligand for the receptor. DeltaC is one of four known members of the Delta family in zebrafish (Haddon *et al.*, 1998). It is likely that there is some compensation for the defects in DeltaC from alternate Notch ligands that would explain the weaker phenotype observed in the BEA than the DES (*Notch1a*) mutants. The defects in myelopoiesis, caused by disruption of Notch signalling, presented here are consistent with the results of Cheng *et al.* (Cheng *et al.*, 2003) who demonstrated that Notch1 deficient ES cells or HPCs had reduced ability to differentiate into myeloid cells. They also support the findings of Schroeder and colleagues who found an increased and accelerated differentiation along the myeloid lineage following co-culture of haematopoietic progenitors with Notch ligand expressing cells (Schroeder *et al.*, 2003). The ability of Notch signalling to enhance myelopoiesis was highlighted recently by Delaney and colleagues in phase 1 clinical trials where substantially enhanced rates of myeloid engraftment were found following conditioning of cord blood cells with Delta-like1 ligands before transplantation (Delaney *et al.*, 2010). The data presented here supports a role for Notch in maturation of myeloid cells, which is in agreement with the findings of Cheng *et al.*, and others, however they have also shown that Notch signalling prevents maturation of immature precursors into mature dendritic cells. In the analysis of WKM cells I failed to observe an increase in the relative numbers of precursors in the Notch mutants. Whilst there was no observed increase in precursor

numbers between WT and Notch mutants, there was a shift towards the lymphocyte lineage in both BEA and DES mutants. There were significantly higher numbers of lymphocytes in the Notch mutant samples than the WT.

Whilst some studies do show a requirement for Notch signalling in the maturation of myeloid cells several studies also present evidence that Notch signalling is required for the maturation of lymphocytes. Stier *et al.* demonstrate Notch1 induced preferential commitment of stem cells to a lymphoid rather than a myeloid lineage, which is in opposition with the findings of this study that a mutation in Notch1 results in preferential development of lymphocytes over myeloid cells (Stier *et al.*, 2002). Notch plays an important role in the differentiation and maturation of T-cells (reviewed by (Radtke *et al.*, 2010). In fact Notch signalling must be tightly regulated and suppressed within the bone marrow in order to prevent ectopic differentiation of T-cells in the bone marrow (Maeda *et al.*, 2007). Several research groups have found that interrupted canonical Notch1 signalling results in inhibition of many cell fate potentials including B-cell and myeloid cell types (reviewed in Radkte *et al.*, 2010). Given that Notch1 signalling can promote the differentiation of T-cells in preference to other cell types does not necessarily disagree with the findings of this study. Here I show mutations in Notch related genes result in the higher proportions of lymphocytes in the WKM. The lymphocyte gate is made up of T-cells and B-cells and therefore it is possible that the impaired signalling in the Notch pathway resulting from the mutations has impaired T-cell differentiation but enhanced B-cell differentiation resulting in greater numbers of lymphocytes overall.

The role played by Notch signalling in cell fate commitments in haematopoiesis is a complex and controversial area of research. The data presented here contributes to this debate and supports Notch signalling as a promoter of the differentiation of the myeloid cell lineage above the lymphoid.

Defect in myelopoiesis has no effect on survival to *M. marinum* infection

Since it has been shown that macrophages play a key role in dissemination and disease progression in mycobacterial infections in zebrafish embryos (Davis *et al.*, 2002a, Clay *et al.*, 2007) I investigated whether or not DES mutant embryos, which have fewer myeloid cells than WT, had altered survival rates in response to mycobacterial infection. Studies in mouse have shown that macrophage depletion during mycobacterial infection results in improved clinical outcome (Leemans *et al.*, 2001, Leemans *et al.*, 2005) however depletion of macrophages in zebrafish embryos resulted in higher bacterial burdens (Clay *et al.*, 2007). In light of these studies some alteration in the rate of mortality between WT and Notch mutant embryos might have been expected. However the reduction in myeloid cells in DES embryos had no effect on survival rates of embryos infected with *M. marinum*. The level of reduction of myeloid cell number in DES embryos was 40% (as assayed by neutrophil response to tail transection) and so 60% of the cells in the myeloid compartment were available for response to infection. This reduction, but not complete depletion, of myeloid cells may explain the absence of an observed effect on survival by the *Notch1a* mutation in the DES fish versus the WT. In a mouse study of mycobacterial infection/macrophage depletion in lung, in which clinical effect of macrophage depletion was observed, 80% depletion of macrophages was achieved (Leemans *et al.*, 2001). Similarly complete depletion of macrophages was achieved in zebrafish mycobacterial infection experiments that resulted in altered disease outcomes (Clay *et al.*, 2007). Perhaps here the partial reduction in myeloid cell numbers was not sufficient to cause an alteration in disease phenotype in this infection model, with the remaining macrophages able to compensate for the overall reduction in number.

CHAPTER 6 – FINAL DISCUSSION AND FUTURE WORK

In this thesis I have extended and characterised existing zebrafish models of inflammation in order to facilitate analysis of host responses to inflammatory signals that differ both quantitatively and qualitatively. Applying these experimental models I have investigated the inhibitory effects of p38 MAPK on different aspects of leukocyte biology, namely transcriptional and migratory responses in zebrafish embryos *in vivo* following wound injury alone and combined with exposure to LPS. The results revealed that only the LPS effect on leukocyte recruitment was p38 MAPK dependent. Also from the analysis of gene expression profiles of inflammatory cells isolated from adult zebrafish I observed that LPS mediated responses are comparable to those reported for mammalian systems. Therefore, in this respect zebrafish mimic mammalian innate responses and may be adapted to provide a high throughput screen to test the anti-inflammatory effects of small molecule inhibitors.

Zebrafish are genetically tractable and their larvae are transparent organisms which has facilitated the development of a novel automated computational single cell tracking and analysis system. This system I have applied in defining the heterogeneity of leukocyte migration to different inflammatory signals. Biological systems available for exploring the dynamical behaviour of leukocytes in real-time and in response to immune modulators are limited and the system I have reported here fulfils both both of these criteria. *In vivo* single cell tracking is still a relatively new and expanding area of biological research. Currently it is possible to track immune migration and cell-interactions in live mouse models. For example using explanted lymph node tissue (Stoll *et al.*, 2002), exteriorised cremaster muscle (Dunne *et al.*, 2004), and by studying inflammatory cellular responses in the iris which is relatively visually accessible (Rosenbaum *et al.*, 2008). Zebrafish, however, have several advantages over existing mammalian *in vivo* leukocyte tracking systems, notably the small size of the zebrafish embryo tail makes it is possible to have the entire region from the transection wound up to the vasculature in the fields of view which means all cells recruited to the wound can be visualised and tracked. Secondly immobilisation of the larvae in agar negates problems caused by

movements such as breathing and eye movements which will affect the mammalian models. While imaging in zebrafish embryos is ideal for studying innate immune cell interactions the lack of an adaptive immune system at this early stage of development prevents analysis of cell interactions between the innate and adaptive immune systems. However, the availability of transparent zebrafish lines (White *et al.*, 2008) may allow imaging to be developed for the analysis of these more complex cellular interactions.

The automated cell tracking system was developed and validated using the effect of LPS on basal leukocyte migration and its sensitivity to p38 MAPK inhibitors as an experimental model. Indeed I was able to detect and quantify the effect of behavioural changes in the dynamic cell recruitment in response to exogenous inflammatory signals. The application of the tracking system in an intact organism allows microenvironments, such as a H₂O₂ gradient on cell migration to exert their physiological effects on cell migration which would be difficult to establish for *in vitro* models. In addition to validating a novel experimental system the results of these experiments have also extended existing knowledge on LPS effects on the dynamics of the migration of single cells gained largely from *in vitro* cell culture studies. The practical application of this study lies in the potential use of this model to determine the efficacy of candidate therapeutics targeting innate immunity in conditions representative of unmodified and infected wound injury.

In light of the potential application of p38 MAPK inhibitors as therapeutic agents for inflammatory diseases such as RA and IBD the primary aim of my thesis was to investigate the mode of action of this class of compounds *in vivo* on the innate immune system. To this end I have demonstrated that p38 MAPK blocks LPS induced leukocyte migration but not that observed following wound injury alone. However, p38 MAPK inhibitors have so far yielded disappointing results in clinical trials designed to evaluate their anti-inflammatory effects. It is thought that a compensatory mechanism abolishes the initial anti-inflammatory effect that occurs *in vivo* and this undoubtedly will involve a combination of innate and adaptive immunity. There is hope however that using less selective inhibitors or targeting kinases higher up the signalling cascade might prove successful alternative strategies. Potential reasons for the unforeseen failure of inhibition of p38 MAPK

inhibitors as therapeutics include dosage limitations (although the more recent compounds do reach therapeutic concentration without toxic effects), bio-distribution (alteration in the lipophilicity of newer drugs to prevent CNS presentation has the potential to limit the efficacy of the compounds), and as p38 α is now known to regulate anti-inflammatory processes (e.g. IL-10 production) the potential anti-inflammatory benefits of inhibition may be counterbalanced (Hammaker and Firestein, 2010). The redundant nature of signalling cascades means that it is likely that effect of the inhibition of one downstream kinase will be compensated by kinases upstream (Hammaker and Firestein, 2010). It is now thought that this might be overcome by targeting multiple kinases higher up in the signalling cascade and the proposed targets are tyrosine kinases including the Janus kinases (JAKs) and spleen tyrosine kinases (Syks). Inhibitors of the JAKs and Syks have been tested in clinical trials and show excellent efficacy in treatment of patients with RA (Hammaker and Firestein, 2010). The cell tracking system presented here provides an *in vivo* screening system for other potential small molecule anti-inflammatory inhibitors and can be used to test the capacity of new alternative strategies to modulate the behaviour of recruited leukocytes.

I investigate leukocyte migration and transcriptional responses in Notch null mutant zebrafish and the results of that analysis confirm other studies that have demonstrated that Notch signalling contributes to the regulation of haematopoiesis and immune responses. The key role played by Notch in cell fate decisions in the bone marrow and its expression in a wide range of tissues restricts consideration of this pathway as a target for the therapeutic modulation of immune responses. However due to its association with the development of some forms of blood cell cancers including leukaemia, for example over 55% of paediatric primary T-ALL tumours tested in one study had at least one mutation in the *NOTCH1* gene (Weng *et al.*, 2004, Radtke *et al.*, 2010) the Notch signalling pathway is being explored as a potential therapeutic target for the treatment of some types of leukaemia. Mutations that induce greater strength of Notch signalling cooperate with other oncogenic hits and influence tumorigenesis (Radtke *et al.*, 2010). The current strategy for targeting inhibition of the Notch pathway in clinical trials is to use γ -secretase inhibitors which non-selectively target all Notch receptors. So far γ -secretase inhibitors have proven

weakly effective against human T-ALL leukaemia in humans and have unwanted side effects including gastrointestinal toxicity (Milano *et al.*, 2004). New small molecule inhibitors that block signalling in the Notch pathway are being developed and early studies indicate some success in blocking the proliferation of leukemic cells *in vitro*. This study has demonstrated that in zebrafish, as in mammals Notch signalling directs cell fate decisions and haematopoiesis. Existing zebrafish models of cancer include models of T-ALL leukaemia (Langenau *et al.*, 2003, Langenau *et al.*, 2005), and could be used to screen the effectiveness of new small molecule for therapeutic effect.

In this thesis I have demonstrated the value of zebrafish to study mammalian immune responses and derived new information on the dynamics of leukocyte recruitment/retention at sites of inflammation. This model organism through its advantages in facilitating optical imaging and genetic tractability I am sure will continue to aid in advancing our knowledge of mammalian biology and human disease.

Future work

There are many directions in which I could expand the results I have described here:

1. An initial extension would be to select other pathway inhibitors and screen their effects on leukocyte migration to wound injury in the presence and absence of other inflammatory signals including LPS.
2. It would be informative to investigate the effect of altering chemoattractant gradients such as H₂O₂ (Niethammer *et al.*, 2009) on the dynamics of leukocyte migration in responses to qualitatively different inflammatory signals.
3. The automated single cell tracking system developed and described in this study currently permits the interpretation of the dynamic behaviour of single cells. I would like to extend the remit of the system to include the transcriptional profile of cells displaying different dynamic behaviour. The gene expression profiling of tracked immune cells could be achieved by the laser dissection and transcriptome profiling of single cells whose dynamical

movement has been previously recorded. This would allow the transcriptional profile of cells to be correlated with their dynamic behaviour and furthermore heterogeneity of behaviour could begin to be associated with specific patterns of gene expression.

4. This *in vivo* cell tracking system could also be expanded to include the interactions of host immune cells with invading pathogens. The recruitment of cells to sites of inflammation that contain fluorescently labelled pathogens would enable the dynamic behaviour of immune cells in the context of live infection to be visualised and analysed. It would also be possible to measure changes in leukocyte migratory behaviour following the phagocytosis of bacteria.
5. It may be possible to adapt the cell tracking system to transparent adult zebrafish to investigate more complex cell interactions between the innate and adaptive immune systems.

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